INFLAMMATION ENHANCED COLON CANCER & NATURAL ANTI-CANCER PLANT COMPOUNDS

BY

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THESIS

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ABSTRACT

Inflammation is associated with an increased risk for colorectal cancer through mechanisms that are not well understood. Isothiocyanates, which are the bioactive components in broccoli and other cruciferous vegetables, have been shown to suppress COX-2 and IL-6 production from inflammation. The polyacetylene gymnasterkoreayne B (GKB) was previously shown to increase detoxification enzymes, but has not previously been tested for anti-cancer activity (1). Here we tested 10% broccoli, 5% broccoli sprouts, GKB and a GKB-rich extract from *Gymnaster koriensis* (GE) for their ability to prevent inflammation-enhanced colon cancer, using the dextran sulfate sodium/ azoxymethane mouse model. GKB (500 μmol/kg diet daily), GE (providing an equal dose of GKB) and broccoli protected against some aspects of inflammation, including cyclooxygenase-2 levels in colonic mucosa. The extract and the 10% broccoli diet, but not purified GKB or 5% broccoli sprouts, protected against colon cancer, decreasing adenocarcinomas by 90%. These data suggest that the Gymnaster extract and broccoli are both promising anti-cancer products.

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CHAPTER 1
INTRODUCTION

1.1 Overview

Rates of colon cancer in developing nations around the world are on a steady rise (2). While developed nations, such as the United States, continue to have the highest rates of colon cancer; countries that are transitioning from undeveloped to developed are experiencing the most marked increase in colon cancer rates (3). According to the National Cancer Institute, the 2008 annual incidence rate of colon cancer per 100,000 in the United States was 34.1 and 25.0 and in South Korea was 46.9 and 25.6 in males and females, respectively (3). Surprisingly, a recent study showed that the United States is the only country that has seen a decline in colon cancer incidence in both males and females (2).

The association between inflammation and cancer began back in 1863 when Rudolf Virchow first discovered leukocytes in neoplastic tissues and determined that the cancer had originated in the presence of chronic inflammation (4). Since then, chronic inflammation has been linked to the development of many different cancers (5, 6). Ulcerative Colitis (UC) is a disease characterized by chronic inflammation of the colon. Patients suffering from UC for several years are at heightened risk of developing colon cancer due to prolonged, chronic inflammation. Indeed, excluding those with genetic abnormalities who sporadically develop colon cancer, patients with UC are at the highest risk of developing colon cancer when compared to other causes (7).

The etiology of colon cancer is very strongly associated with genetic and environmental influences (8, 9). Thus, the increase of colon cancer seen in economically transitioning nations is often attributed to changes in diet and lifestyle, characterized by diets low in fiber, high in meat protein and refined carbohydrates and an increasingly sedentary lifestyle leading to obesity (3).

While dietary interventions have been somewhat inconclusive, regular physical activity has been shown to reduce risk of developing colon cancer. Interestingly, one can improve clinical outcomes by becoming physically active even after diagnosis (10-12). Bioactive components of broccoli, particularly sulforaphane, have been implicated in their ability to ameliorate inflammation (13-18). Studying the impact of dietary intervention on inflammation associated colon cancer, it is important to elucidate what role diet may play in the disease process and whether dietary cancer preventive methods are possible.

1.2 Hypotheses & Aims

Inflammation pilot study 1

Hypothesis: There will be a difference in the signs of inflammation observed in mice receiving 3% Dextran Sulfate Sodium (DSS) in their drinking water for 5 and 7 days. The severity of inflammation in the 7 day model will be greater than that of the 5 day model.

Aim 1: Establish a method to measure severity of inflammation using the Disease Activity Index and histopathological analysis of small intestine and colon.

Aim 2: Determine appropriate dosage of DSS in mice so that the mouse model of acute inflammation in the colon can be used in future studies.

Inflammation pilot study 2

Hypothesis: Mice receiving 1% DSS in their drinking water for 5 days will experience more severe inflammation than mice receiving this dose for 3 days. Mice will recover from inflammation after a 7-9 day period of plain drinking water after DSS treatment as evaluated by histopathology.

Aim 1: Re-evaluate appropriate dose of DSS to produce acute inflammation without progressing to morbidity or mortality in mice. Determine whether this dose can be used in a long term inflammation enhanced colon cancer study.

Rappini inflammation study

Hypothesis: Mild to severe inflammation will be seen in mice receiving 1% DSS in their drinking water for 5 days on the standard AIN-93G diet. Colon sections of these mice will reveal the presence of numerous inflammatory cells, edema and shortened crypts.

In mice receiving 1% DSS in their drinking water, plus a10% broccoli or rappini diet, inflammation will be ameliorated and colon sections will appear normal, with the absence of inflammatory cells, edema, and shortened crypts.

Aim 1: Compare dietary broccoli and rappini for efficacy at reducing or mitigating acute inflammation induced by DSS in C57bl/6 mice as evaluated by histopathology.

Inflammation enhanced colon cancer study

Hypothesis: Broccoli sprouts rich in sulforaphane, broccoli powder, as well as extracts from Korean aster (A164-a and D FR-11) when consumed as part of the diet will provide protection against dextran sulfate sodium/ azoxymethane-induced colon cancer in C57bl/6 mice, reducing tumor promotion and progression.

Aim 1: To test the cancer-preventive activity of one promising compound, and an extract containing that compound, from a Korean aster plant, as well as 10% dietary broccoli and 5% broccoli sprouts, in a mouse model of colon cancer.

Aim 2: Determine safety of long term treatment of dietary components, (broccoli and broccoli sprouts powder, Korean compound and fraction), by pathological observation and biochemical evaluation of liver and other organs following necropsy.

CHAPTER 2
LITERATURE REVIEW

2.1. Physiology & Function of the Colon

The colon comprises the greatest portion of the large intestine which is approximately 1.5 meters long including the cecum and rectum. The colon consists of four segments which are the ascending, transverse, descending and sigmoid colon. These segments are responsible for absorbing the remaining electrolytes and approximately 500-1000mL of water per day which the small intestine has failed to absorb. Any water that is not absorbed in the colon (~50-200mL) is excreted in the feces. Other functions of the colon include storage and bacterial synthesis of vitamins, namely vitamin K, B^{12}, thiamin, and riboflavin (19).

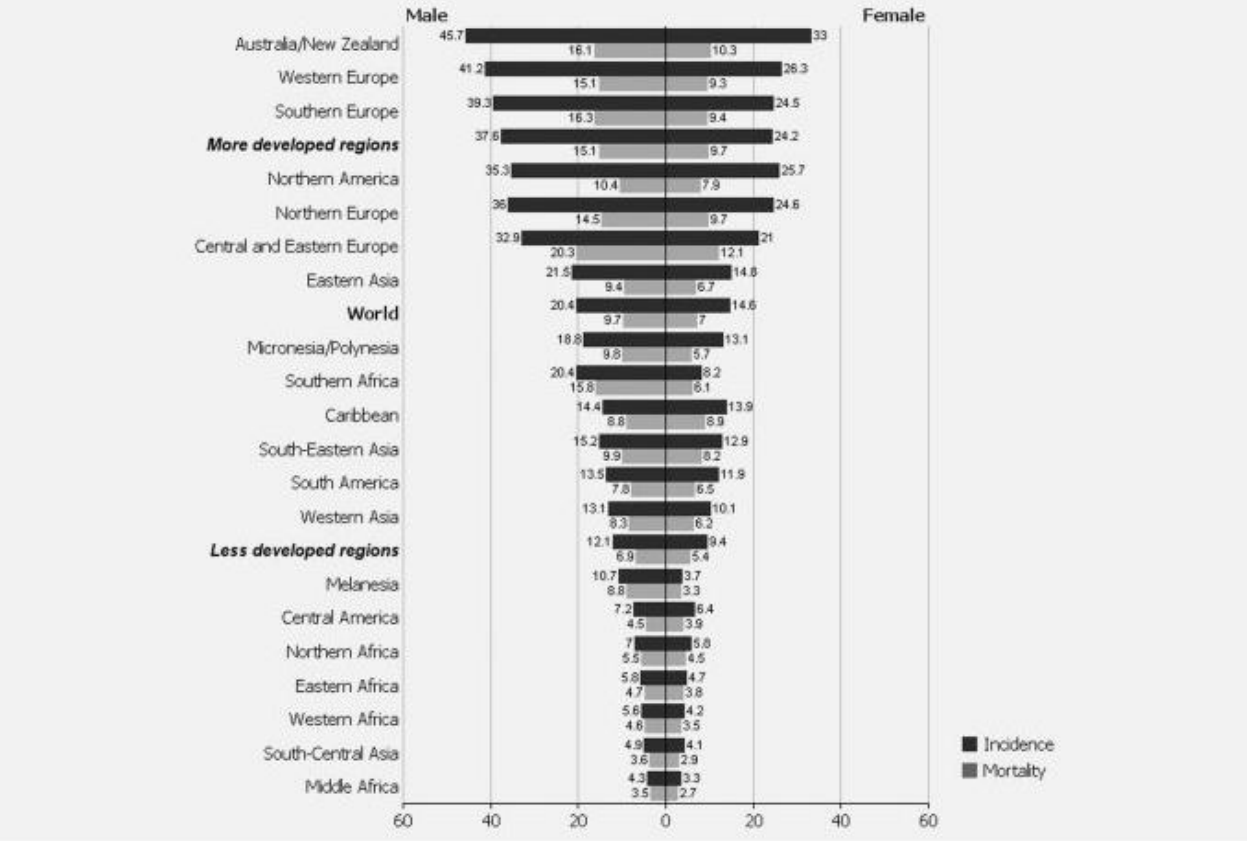
Fermentation is another major function of the colon and occurs when insoluble fiber, resistant starches, undigested amino acids and mucus are fermented by resident bacteria, producing gases and short chain fatty acids (SCFAs). In return, SCFAs serve as an energy source to increase colonocyte cell proliferation and differentiation, increase water and electrolyte absorption and decrease osmolality. The microflora of the colon is thought to be comprised of more than 500 bacterial species, of which 75% remain uncharacterized (20). The process of colonic bacterial fermentation, especially how this process may be modulated by diet, has been a source of great interest in research and in commercial food development. Prebiotics and probiotics in the diet are thought to increase SCFAs and consequently lead to an increase in healthy resident bacteria, conferring a benefit to the host in warding off disease (21, 22). In addition, the bacteria deconjugate dietary components (such as isoflavones), secreted xenobiotic metabolites (such as acetaminophen glucuronide) and conjugates of endogenous components (such as estrogen conjugates) creating products available for absorption (23-25).

2.2 Colon Cancer

Colon cancer is the third most common type of cancer diagnosed in the Western world. It is the second leading cause of cancer death in the United States and the fourth leading cause of cancer death in Korea (26, 27). It is estimated that there are 108,070 new cases of colon cancer in the United States each year (139). Incidence rates in males are higher than in females, but colorectal cancer is the second most common type of cancer in women while it is the third most common in men (28). More developed regions have higher incidences and mortality rates of colorectal cancer than less developed regions (Figure 1). Colon cancer is significantly more prevalent in African Americans when compared with Caucasian Americans suggesting a socioeconomic factor. Some people may have a genetic predisposition to developing colon cancer such as Familial Adenomatous Polyposis or Hereditary Nonpolyposis Colorectal Cancer (29). However, epidemiological studies have estimated that only 15% of colorectal cancer cases are caused by these genetic defects. Hence, the etiology of colon cancer is complex and multifaceted. Colon cancer has been linked to genetic predispositions, environmental triggers, including infectious causes, and inflammatory disease, such as Crohn's Disease and Ulcerative Colitis (3, 30-33).

A recent cohort study using a North American population revealed that compared to non-Inflammatory Bowel Disease patients, there was an increased incidence rate of colon carcinoma for Ulcerative Colitis (UC) patients within similar geographical locations (34). The risk of developing cancer for UC patients increased with age, estimated at 7-14% greater risk after 25 years and up to 30% after 35 years. Within the same study, the increased risk of colon carcinoma was also higher in males when compared to females (34).

Figure 1. Estimated age-standardized incidence and mortality rates for colorectal cancer.



Source: (28)

2.3 Inflammatory Bowel Disease: Ulcerative Colitis

Inflammatory Bowel Disease (IBD) involves chronic inflammation of the gastrointestinal tract and includes two forms: Crohn's Disease (CD) and UC. The prevalence of IBD is ~100 to 130 cases per 100,000 and the incidence is ~4 to 10 new cases per 100,000 annually. There is no difference in incidence rates between males and females but some populations have a greater risk including the United States, United Kingdom and Scandanavian countries (35). Onset of disease commonly occurs across a broad age range, from 15 to 80 (36). Many symptoms are common

between CD and UC, including diarrhea, food intolerances, anemia, malnutrition as well as extra-intestinal manifestations such as weight loss, fever, renal disease, bone abnormalities, hepatobiliary disease, oral ulcers and arthritis (35). UC is also characterized by blood in the stool. This is a clinical sign which is often used to differentiate between UC and Irritable Bowel Syndrome; which is a disorder featuring recurrent abdominal pain and diarrhea thought to arise from emotional stress. Whereas CD can also present with bloody stool and thus can be misdiagnosed as UC, it often has more subtle symptoms which can delay a proper diagnosis. The main differences between CD and UC are in where and how they affect the GI tract. In CD any segment of the GI tract may be affected and inflamed areas may be separated by normal areas. In addition, CD is transmural and often extends to the submucosa and serosa resulting in fistula formation, abscesses, fibrosis, submucosal thickening and possible obstruction of the bowel. In contrast, the inflammation in UC is restricted to the colon and occurs in a continuous area without being separated by any areas of normal colon. Furthermore, UC only affects the mucosal layer and often results in bleeding, ulceration and edema (35, 37). Since the animal model we use mimics human UC, I will focus on UC instead of CD in the remainder of this literature review.

Pathogenesis of UC:

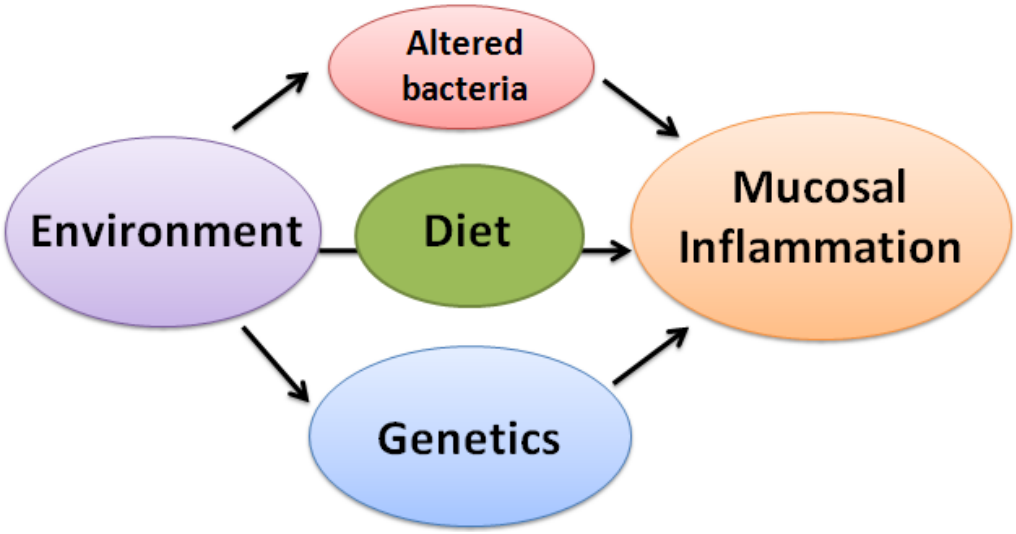
The cause of UC is not well understood but it is thought to be a result of genetic and environmental factors coupled with an overactive immune response (Figure 2). More specifically it is thought that the mucosal immune system becomes dysregulated, which can cause an inflammatory response from a usually harmless source to expand and persist and negatively affect the normal microbiota (36, 38). The disease process of IBD is broken down into three phases – initiation, augmentation and perpetuation. Initiation involves the environmental triggers

that can affect the genetically susceptible individual, augmentation involves the innate and adaptive immune response, and perpetuation involves the regulation of the immune response. The initiation and augmentation phases involve both the innate and adaptive immune response, while the perpetuation phase involves only adaptive immunity.

The innate immune response in the colon involves both the epithelial barrier and the phagocytic cells within the lamina propria, which are the macrophages, dendritic cells (DCs), lymphocytes, plasma cells and neutrophils. It has been noted that patients with genetic abnormalities in innate immunity have a higher risk of IBD.

The adaptive immune response is involved in immune regulation, which in the gut is typically immune suppression, which protects commensal microbiota from immune responses. The immune cells involved include T cells, B cells, DCs, macrophages and NK T cells. In particular, T inducible regulatory type 1 (Tr1) cells and T helper 3 (TH3) cells are implicated as secreting interleukin-10 (IL10) and transforming growth factor β (TGFβ) respectively, which are cytokines involved in strong immunosuppression. The absence of IL10 in mice leads to colitis characteristic of Crohn's Disease, demonstrating its potency. The current hypothesis for IBD is that defective regulatory T cells, specifically Type 2 CD4+ T cells, cause the perpetual activation of the immune response, leading to the chronic inflammation seen in IBD (39).

Figure 2. Etiology of Ulcerative Colitis, Adapted illustration (39)



Diagnosis of UC:

The first step in diagnosing UC is to rule out other causes of chronic diarrhea such as Irritable Bowel Syndrome (IBS) and other forms of colitis, such as ischemic, pseudomembranous, infectious colitis and Crohn's Disease. Suspected cases of UC can be further investigated by examining the stool for alternative causes of diarrhea. Also laboratory tests such as complete blood count can check for anemia secondary to blood loss and electrolyte abnormalities such as hypokalemia secondary to diarrhea. Erythrocyte sedimentation rate and C - reactive protein may also help with diagnosis as they are often elevated in UC due to systemic inflammation. Colonoscopy and biopsy are the main ways of diagnosing UC and differentiating from CD by comparing features of the diseases, such as depth and location of inflammation. For

those with established UC, the physical examination and extra-intestinal manifestations can aid in determining the severity of the disease and the course for treatment (36).

Treatment of UC:

The two areas of treatment for the patient with UC include managing the acute symptoms and maintaining remission. The most common therapy is 5-aminosalicylic acid (5-ASA) which works to reduce the immune response by suppressing cytokines and other proinflammatory mediators. When patients do not respond to 5-ASA or it is contraindicated, then the next option is oral steroids, usually prednisone. Oral steroid usage should be limited to controlling acute symptoms, usually one to two weeks, because of negative side effects. If patients do not respond to steroids, then hospitalization involving more aggressive treatments may be required until symptoms subside. Other common medications for UC include antibiotics and immunomodulators, such as azathioprine or 6-mercaptopurine. More novel treatments include probiotic therapies, curcumin, and infliximab (anti-TNFα). It has also recently been shown that cessation of smoking can increase the risk of UC, although there is no difference in incidence between smokers and non-smokers. In severe cases of UC or when the patient has developed low grade dysplasia or cancer, then colectomy is performed in order to officially eradicate the disease (40). While surgery often improves the patient's quality of life dramatically, it can also result in complications such as pouchitis, bowel obstruction and stricture (36, 38).

Mechanism of Colitis Associated Cancer Development:

When the chronic inflammation of UC is prolonged and intense enough, the risk of developing colon cancer is greatly increased. All stages of cancer development are susceptible to and can be driven by inflammation. Reactive oxygen and nitrogen species, released by cells

within the innate immune response, can cause DNA mutations, thereby affecting the tumor initiation phase. During tumor promotion and progression nuclear transcription factor kappa B (NF-κB) within CD4+ T cells is thought to promote the release of interleukin-6 (IL6) and tumor necrosis factor (TNF)-α. The receptors which bind IL6 and TNF-α can activate downstream targets, such as the Signal Transducers and Activators of Transcription (STAT) pathways. In particular, the STAT3 pathway is thought to be intimately involved in the promotion of colitis associated colon cancer by promoting tumor proliferation and survival (41).

Cyclooxygenase-2 (COX-2) enzyme activity and prostaglandin E_2 (PGE_2), a product of COX-2 stimulation, are also thought to play an important role in tumor promotion and progression. These pro-inflammatory proteins are thought to lead to increased angiogenesis and metastasis, abnormal immune modulation and suppression of apoptosis. Elevated expression of COX-2 can be seen in patients suffering from UC and in 85% of patients with colorectal cancer (42).

2.4 Diet, Inflammation & Cancer

The causes of cancer have been largely attributed to genetic and environmental factors, including lifestyle, and are generally thought of as either avoidable or unavoidable. Dietary habits have been considered for years in epidemiological and case controlled studies to have an impact on cancer development and prevention (43). However, this association between diet and cancer has never been as clear as the correlation between smoking and cancer. Overnutrition, leading to obesity, has also been associated with increasing cancer development in many animal studies and is also considered a risk factor for many types of cancer (44). Similarly calorie restriction appears to decrease risk for many cancers (45). A major focus of diet and cancer

research pertains to individual dietary components that may reduce or enhance cancer risk. Studying dietary components also gives more insight into the mechanisms involved in cancer development and how diet may play a role in modulating these mechanisms. Some examples of dietary factors that may enhance or prevent cancer are outlined in tables 1 and 2 respectively.

Table 1. Dietary components that may enhance cancer risk		Sources
2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)	Carcinogens present in red meat and processed meat are known to increase the risk of developing colorectal cancer. A small clinical trial showed that a diet high in cruciferous vegetables (broccoli and Brussels sprouts), can increase excretion of PhIP by induction of detoxification enzymes suggesting one mechanism by which broccoli reduces colon cancer risk.	(46)
Alcohol	Cancer of the oral cavity, pharynx, larynx, esophagus, liver, colorectum and female breast are considered inversely related to increased alcohol consumption. The mechanism is unclear but may involve increased reactive oxygen species and/or support of the Warburg effect, which is the change in energy production associated with decreased NAD/NADH.	(47)
Beta carotene	Two clinical trials have shown that beta carotene supplements increase the risk of lung cancer in smokers. Eating fruits and vegetables containing beta carotene is associated with cancer reduction but this has not been shown to be attributed to beta carotene. Furthermore, the dose of beta carotene which enhanced cancer risk was two orders of magnitude greater than normal blood beta carotene.	(48)
Natural carcinogens	Aflatoxin is produced from *aspergillus flavus* on corn and other crops, including peanuts; this fungus grows particularly well in hot, humid climates. Aflatoxin is a cause of liver cancer.	(43)

Table 2. Dietary components that may decrease cancer risk		Sources
Fruits and vegetables	There is enough evidence from epidemiological studies to warrant a recommendation by the FDA that consuming at least 5 servings of fruits and vegetables daily may decrease risk for cancer.	(49) American Cancer Society
Phytochemicals	Many dietary compounds possess cancer preventive properties including, curcumin, luteolin, resveratrol, genistein, lycopene and sulforaphane which act by various mechanisms.	(50) (51)
Antioxidants	Most plant phytochemicals are also antioxidants *in vitro*. Polyphenolic compounds have been of particular interest due to their antioxidant properties such as those from green tea, including epigallocatechin-3-gallate, and berries, including proanthocyanidins.	(50)
Calcium	Controlled clinical trials have shown a moderate reduction of colorectal cancer with intake of calcium supplements, but the evidence is inconclusive.	(52)
Folate	There is conflicting evidence which indicates that folate can be protective against cancer in early stages of cancer but possibly enhance cancer in later stages.	(53)
Selenium	Epidemiological evidence has shown that selenium has a significant impact on reducing cancer risk, especially prostate cancer. A clinical trial in which the primary focus was skin cancer, found decreased prostate cancer risk with ingestion of selenized yeast. Protection was not repeated in the SELECT trial.	(54) (55)
Vitamin E	Many studies have shown that vitamin E supplementation can protect against prostate cancer. Some have suggested that vitamin E supplements be used in prevention of prostate cancer in men at high risk. Protection was not repeated in the SELECT trial.	(56)

2.5 Animal Model Selection

We chose to use the Dextran Sulfate Sodium (DSS) method as an IBD model because it can be given in the water *ad libitum,* has a low rate of mortality and most importantly it mimics human UC in that it only affects the colon (57). Since IBD is a chronic condition we chose the C57bl/6 mouse because it was recently shown that DSS administration (5 day regimen of 1% DSS in drinking water) creates a chronic state of inflammation in this strain as opposed to an acute injury from which the mouse can recover (58). We ultimately chose a dosing regimen of 1% DSS in drinking water for 7 days based on a pilot study we performed (See methods, Chapter 3 – Pilot 2). Further validation of the DSS model was shown in a study using C57bl/6 mice in which they were given common human IBD drug therapies which reduced local cytokine levels and improved disease symptoms and histology, proving that this model truly reflects the human disease (59).

Our inflammation-driven cancer model adapted from Suzuki, using one dose of azoxymethane (10 mg/kg mouse i.p. injection) followed by a one week rest and then a one week treatment of 1% DSS was chosen because it reflects how human chronic IBD can lead to colon cancer. In this model the C57bl/6 mouse was shown to have an incidence of colonic adenocarcinomas of 50% with a multiplicity of 1.0 ± 1.2 (60).

2.6 Cruciferous Vegetables and Cancer

Epidemiological evidence and case control studies suggest that there is an inverse relationship between increased cruciferous vegetable intake and different types of cancer, including lymphoma (61), lung (62, 63), colorectal (64, 65), prostate (66), breast (67), and gastric cancer (68). Cumulatively, these studies suggest that eating 3-5 servings of broccoli a

week can reduce the risk of developing various forms of cancer (13). Epidemiological research has also identified the important role of genetic interactions in the diet-cancer relationship. In individuals possessing low glutathione-*S*-transferees activity, a diet high in cruciferous vegetables has been shown to significantly reduce the risk of developing colorectal cancer. These individuals have both GSTM1 and T1 null genotypes, indicating that ITC compounds are not metabolized and excreted efficiently and remain in the body providing protection against cancer (64). However, this research has been contradicted in several other studies which show that GSTM1 positive individuals are better protected against cancer when consuming a diet high in crucifers (69).

Clinical studies – Clinical studies have investigated the safety and efficacy of reducing cancer risk using cruciferous vegetables, including broccoli and broccoli bioactive components. Sulforaphane, which occurs as the glycoside glucoraphanin within broccoli, has been studied extensively due to its multiple anticancer properties. Only one small clinical trial (n=28) has investigated the effect of broccoli on colon cancer using induction of Glutathione-*S*-Transferase (GST) as a measure of efficacy. While the dose of broccoli had no significant effect on GST induction over the control, it was shown that GST induction in blood lymphocytes had a significant correlation ($r_s = 0.71$) with that in colon mucosa (70). Induction of phase II detoxification enzymes, NAD(P)H:quinone oxidoreductase and heme oxygenase-1, was observed in mammary tissue after a single treatment of 200 μmol sulforaphane (SF). This study confirmed that SF does distribute to and can be measured in breast epithelial tissue (71). In a second study, 68 grams of broccoli sprouts were able to significantly inhibit histone deacetylase (HDAC) activity in human peripheral blood mononuclear cells. Inhibition of HDAC activity is thought to be an anticancer mechanism because HDAC is known to be greatly increased in

several forms of cancer (72). Another study observed the effects of broccoli on male smokers and non-smokers with a 200g dose of broccoli given for 10 days. While HDAC activity was not found to change, DNA damage measured as DNA strand breaks in lymphocytes was significantly decreased with broccoli treatment (73). Another study examined the safety of consuming broccoli sprouts extracts containing 25 or 100µmol glucosinolates or 15µmol isothiocyanates. After administration for 7 days (21 doses), no adverse effects were found during analysis of blood or urine (74). In a study looking at bacterial communities in the colon, a 14 day diet rich in cruciferous vegetables was able to change the bacterial composition of each participant when compared to the control diet lacking in fruits and vegetables. However, each individual responded uniquely to the high cruciferous vegetable diet, which may help illuminate individual exposure to isothiocyantes after gut fermentation and help predict cancer risk (75).

Animal studies – Studies conducted in animals provide a more profound understanding of the mechanisms involved in the cancer protective effects of cruciferous vegetables and their bioactive components. *Apc*^{min} mice have a genetic mutation which causes them to develop many intestinal tumors at an early age. These mice were used to investigate the effects of SF on intestinal tumor development. Long-term treatment of mice with SF increased levels of acetylated histones in the colon and suppressed tumor multiplicity (76). Another long-term SF feeding study using *Apc*^{min} mice resulted in significantly fewer polyps in the small intestine, with increased apoptosis and decreased proliferation indices. Treatment with SF also significantly decreased the expression of pro-survival signalling pathways, including phosphorylated c-Jun N-terminal kinase, phosphorylated extracellular signal-regulated kinases and phosphorylated-Akt, when compared to *Apc*^{min} mice without SF treatment (77). Cancer in multiple other organs has

also been diminished by feeding broccoli (78, 79) or administering sulforaphane (80, 81) as demonstrated in various animal models.

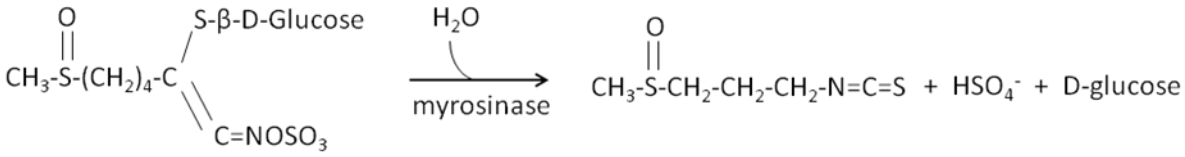
2.7 Anti-Cancer Effects of Glucosinolates and Hydrolysis Products

Cruciferous vegetables are lauded for their health promoting properties. Some common vegetables of this variety include broccoli, Brussels sprouts, kale, collard greens, cauliflower, cabbage and watercress. The secondary metabolites within these plants exist as a result of the plants' natural defense system. One such group of secondary metabolites known as glucosinolates (GS) consist of a β-D-thioglucose group, a sulfonated oxime group, and a variable side chain. There are over 120 glucosinolates already identified in various crucifers (82). Glucosinolates are converted to bioactive isothiocyanates (ITC) in the presence of water and myrosinase, an endogenous enzyme within the plant. Once the plant matrix has been disrupted by chewing or crushing and mixed with saliva or water, myrosinase is released from the plant tissue and able to break down GS into ITC. (See table 2.2). In the absence of myrosinase, GS can be broken down less efficiently in the colon by resident microbiota (83, 84). Nitriles, epithionitriles, thiocyanates or oxazolidine-2-thiones are formed depending on the presence of myrosinase-associated proteins, such as epithiospecifier protein. These nitriles can also be formed at low pH and in the presence of Fe^{++} ions *in vitro*; however nitriles are typically not bioactive compounds. After absorption, isothiocyanates, such as sulforaphane, are conjugated with glutathione via the mercapturic acid pathway and excreted as N-acetylcysteine conjugates (85).

The major glucosinolate groups that exist in brassica vegetables are indole, aliphatic and aromatic. Aliphatic GS in crucifers mainly include glucoraphanin, glucoiberin, sinigrin, and

progoitrin; indole GS include glucobrassicin, neoglucobrassicin, methoxy-glucobrassicin, and 4-hydroxy-glucobrassicin. The most studied isothiocyanate is sulforaphane (1-isothiocyanato-4-methylsulphinylbutane) which is derived from glucoraphanin (Figure 3) and thought to act as an antioxidant, anticancer, antimicrobial and anti-inflammatory compound when ingested (14).

Figure 3. Conversion of the glucosinolate glucoraphanin to the isothiocyanate sulforaphane in the presence of myrosinase and water.



Glucoraphanin Sulforaphane

Antioxidant activity of sulforaphane from broccoli:

Broccoli is a good source of many nutrients, including vitamin C and E, and phytochemicals, particularly isothiocyanates, which give it many health promoting properties. Broccoli also has antioxidant properties which can be attributed in part to its polyphenolic content, including quercetin and kaempferol (86). Vitamins C, E, and beta carotene also play a part in antioxidant properties (87). Long-term, isothiocyanates can upregulate a number of antioxidant enzymes. Sulforaphane can act indirectly as an antioxidant by increasing the levels of tissue glutathione and thioredoxin reductase which can help remove reactive oxygen species (88, 89).

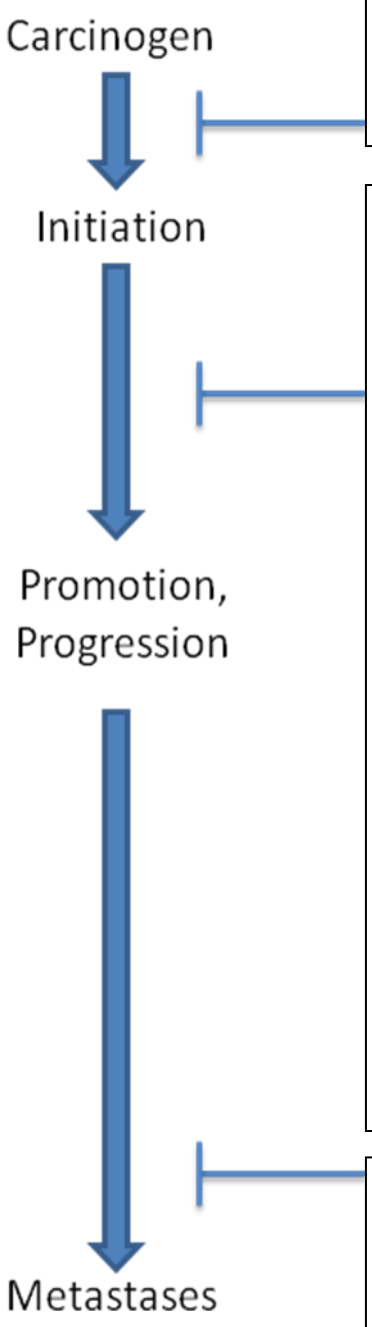
Anticancer activity of sulforaphane from broccoli:

Broccoli contains multiple compounds which may provide protection against cancer in ways other than antioxidant capacity, including indole and aliphatic isothiocyanates. Sulforaphane

(SF), an aliphatic isothiocyanate, has the potential to work by various mechanisms to prevent the initiation and thwart the progression of cancer (Figure 4). One of the earliest discoveries, in the 1980s, was that SF could upregulate detoxification enzymes in the liver, including the phase II enzyme glutathione-*S*-transferase (90), and then in 1992 quinone reductase was shown to be upregulated (91). Phase II enzyme induction via SF-dependent ARE-mediated transcription is thought to help clear carcinogens from the body and decrease the insult caused by ROS, thus preventing cancer initiation (13, 83).

Detoxification of carcinogens by enzyme induction is only one of the multiple mechanisms by which SF has been shown to act. Sulforaphane is also involved in apoptosis, which is the elimination of damaged and unnecessary cells (14, 92)

Figure 4. Sulforaphane acts at multiple sites in cancer prevention depending on the stage and location of cancer.



Detoxifcation enzyme modulation: SF inhibits phase I detoxification enzymes, such as cytochrome P_{450} 1A1, and enhances phase II detoxification enzymes, such as quinone reductase. However, some phase I enzymes, such as CYP4A10 can be increased as a protective mechanism.[13, 82,92]

Cell Cycle Arrest: SF can modulate the phosphorylation of cyclin dependent kinases (CDK), cyclins and CDK inhibitors possibly through the mitogen-activated protein kinase signaling pathway and thereby arrest cell cycle progression. Different phases of the cell cycle are susceptible to SF, including G_1 and G_2/M. It is thought that the cell cycle inhibitor protein p21 plays a role in SF mediated cell cycle arrest.[92, 94]

Apoptosis: SF inhibits cancer by induction of apoptosis, by increasing poly (ADP-ribose) polymerase cleavage and increasing pro-apoptotic Bax proteins and decreasing anti-apoptotic Bcl proteins. Also, by conjugation with glutathione during its metabolism, SF increases reactive oxygen species thereby disrupting the mitochondrial membrane and causing apoptosis.[13, 82, 94]

Cell proliferation: By inhibiting the nuclear factor kappa B transcription factor or through the Nrf2 pathway by disruption of AP-1 binding, SF can inhibit cell proliferation reducing the progression of cancer.[13, 82]

Histone deacetylase (HDAC) inhibition: HDAC, an enzyme which removes acetyl groups from N-acetyl lysine amino acids on histones, is thought to be involved in the transcriptional regulation of cell differentiation, apoptosis and cell cycle progression and is increased in cancer development. SF has been shown to inhibit HDAC activity in prostate and colon cancer cell lines as well as in *APC*^{min} mice.[76, 82]

Angiogenesis: SF has been shown to inhibit angiogenesis *in vitro* by reducing capillary –like tube formation and cell migration. These changes were shown to be attributed to angiogenesis growth factors, such as vascular endothelial growth factor (VEGF) and VEGF receptors, as well as c-myc and matrix metalloproteinase-2. In vivo studies have also shown that SF can inhibit VEGF.[93]

2.8 Anti-inflammatory properties of sulforaphane from broccoli

A known risk factor for developing colon cancer is chronic inflammation. As mentioned previously, Inflammatory Bowel Disease can often lead to colorectal cancer, the risk ranging from 0.9 to 8.8-fold in patients with Ulcerative Colitis (95). Chronic inflammation leads to the up-regulation of pro-inflammatory enzymes, such as COX-2, which can lead to the increased expression of prostaglandins. Elevated levels of prostaglandins have been associated with decreased apoptosis, and increased proliferation and metastasis of cancer cells (92). Many studies have observed that anti-inflammatory drugs and some dietary compounds are capable of reducing inflammation in human UC, as well as in animal models of Colitis-Associated Colon cancer (33, 77, 96-99).

Sulforaphane may also impact colon carcinogenesis through inhibition of inflammation by affecting detoxification enzymes and by impacting genes in the inflammation pathway. Recent studies in *Apc*^{min} mice have revealed that sulforaphane can reduce inflammation and thus reduce colon tumorigenesis (77, 100). A potential mechanism by which sulforaphane can act as an anti-inflammatory is through the Nuclear Factor erythroid 2-Related Factor 2 (Nrf2) pathway, which regulates the antioxidant response element important in ameliorating oxidative stress. Since oxidative stress plays an important role in cancer development, anything that may alter the Nrf2 pathway is a target of interest in cancer prevention. Sulforaphane also is known to inactivate the pro-inflammatory transcription factor Nuclear Factor kappa B (NF-kB), resulting in the reduced induction of COX-2 and inducible Nitric Oxide Synthase (iNOS) expression, as well as decreased TNF-α secretion (92). In another study, sulforaphane was effective at increasing production of two anti-inflammatory cytokines thought to reduce cancer risk, IL-2 and interferon-γ, in tumor-bearing Balb/c mice (101). A couple of studies have also demonstrated

that sulforaphane may reduce inflammation by induction of the phase II detoxification enzyme, NQO1 (102, 103).

CHAPTER 3
INFLAMMATION PILOT STUDIES

3.1 Introduction: Inflammation pilot study 1

The purpose of this pilot study was to establish the mouse model of acute inflammation in the colon so that it could be used in future studies in our lab group. Further investigations of how diet can impact inflammation and how the diet-inflammation interaction can impact cancer development have subsequently been performed. After a review of the literature we found that a common method of studying inflammation in rodents is to treat with Dextran Sulfate Sodium (DSS) dissolved in the drinking water. One research group had shown that after 5% DSS the C57bl/6 mice experienced inflammation that was too severe. However, they have shown that 3% DSS given for 5 days was able to produce less severe inflammation and mice carried out to 30 days only had mild inflammation (58). We developed our model based on this information.

3.2 Methods

Animal Care:

Eight to ten week old male C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed individually in shoebox cages at the Institute for Genomic Biology animal facility at the University of Illinois (Urbana-Champaign) and maintained under 12-hour light/dark cycles at 22˚C and 60% humidity. All animals were allowed water and AIN-93G diet *ab libitum*. After a 3 day period of acclimation mice were divided into 5 groups: group I, 3% DSS in drinking water for 5 days; group II, 3% DSS in drinking water for 5 days and 2 days recovery; group III, 2% DSS for 5 days; group IV, 2% DSS for 7 days; group V, control. All animal care followed the approved protocol by the Institutional Animal Care and Use

Committee and the Division of Animal Resources at the University of Illinois, Urbana-Champaign, in accordance with NIH regulations.

Reagents:

All diet ingredients were ordered from Harlan-Teklad laboratories (Madison, WI) and mixed following the Harlan-Teklad standard AIN-93G recipe (Table 3). Dextran sulfate sodium (M.W. 36,000-50,000kDa) was purchased from MP Biomedicals, LLC (Solon, OH).

Table 3. AIN-93G standard composition

Formula	g/Kg
Casein	200.0
L-Cystine	3.0
Corn Starch	397.5
Maltodextrin	132.0
Sucrose	100.0
Soybean Oil	70.0
Celluose	50.0
Mineral Mix	35.0
Vitamin Mix	10.0
Choline Bitartrate	2.5

Website:
http://www.harlan.com/research_models_and_services/laboratory_animal_diets/teklad_custom_research_diets/ain_formulas.hl

Experimental Design:

Mice were allowed 3 days acclimation before administering DSS in drinking water. Three percent (w/v) DSS was dissolved in de-ionized, distilled water and given fresh daily to mice for 5 days (n=6) and 3 days + 2 days recovery on regular tap water (n=6). Two percent (w/v) DSS was given to mice for 5 days (n=3) and 5 days + 2 days water (n=3). Control mice

received regular drinking water without DSS throughout study (n=1). At the end of the DSS treatments, mice were anesthetized using ketamine/xylazine (87mg/mL and 13mg/mL respectively at 0.1mL/100g BW). Blood was drawn by cardiac puncture and mice were killed by cervical dislocation without recovery from anesthesia. Livers were immediately perfused with ice-cold KCl solution (1.15% w/v) and weighed. The colon was removed distal from the cecum to distal from the rectum, flushed with ice-cold KCl solution, measured, and the mucosa was scraped and collected. All samples were snap frozen in liquid nitrogen and stored at -80˚C until further processing.

Monitoring of Overt Signs:

Mice were monitored daily for observable changes in behavior and inflammatory signs. Percent weight loss, stool formation and presence of fecal blood, if observed in mice, were given scores on a scale developed as a disease activity index (Table 4). Mice experiencing severe inflammation were monitored closely by a veterinary technician within the facility and were euthanized early if they did not recover. The original experimental design was to administer 3% DSS for 7 days. Groups were reformulated by reducing the number of days to 5 days and 3 days after mice on the 3% DSS regimen showed severe signs of inflammation. After the groups were separated, the number of mice in the control group was reduced to one.

Table 4. Disease Activity Index (scoring 0-9)[1]

Score	Weight loss	Stool formation	Fecal bleeding
0	None	Normal pellet	None
1	1-5%	Loose stool (mild)	Blood in stool (mild)
2	5-10%	Loose stool (moderate)	Blood in stool (moderate)
3	>10%	Watery diarrhea	Gross bleeding

*Normal stools = well formed pellets
*Loose stools = pasty stool
*Diarrhea = liquid stool
*Mild = intermittent, Moderate = frequent

[1]Mice were given a score of 0-3 for each of the three categories and the sum was used to determine the stage of colitis as follows:
1-4: Mild colitis (example: 1 for wt loss, 1 for stool and 0 for bleeding = score of 2)
5-7: Moderate colitis
8-9: Severe colitis

Histopathologic examinations:

At autopsy, the colon was removed distal from the cecum to distal from the rectum and flushed with 1.15% potassium chloride (w/v). The length was measured and sections were cut from the distal and proximal colon and fixed in 10% neutral buffered formalin. After 24 hours, sections were washed and placed in 80% ethanol and stored at 4˚C until further processing at the Veterinary Diagnostic Laboratory (University of Illinois, Urbana, IL). Fixed tissues were embedded in paraffin and 10 micrometer cross-sections were mounted and stained with hematoxylin and eosin (H&E). Slides were examined using an Olympus BX51 microscope, an Olympus DP70 three-chip camera and an Antec EN8900 series computer for storage of images.

Statistical Analysis:

Values are means ± SEM. Data was analyzed using ANOVA; post hoc Tukey's test for multiple comparisons on SAS statistical software (SAS Institute Inc., Cary, NC). $P \leq 0.05$ was considered significant.

3.3 Results of Pilot 1

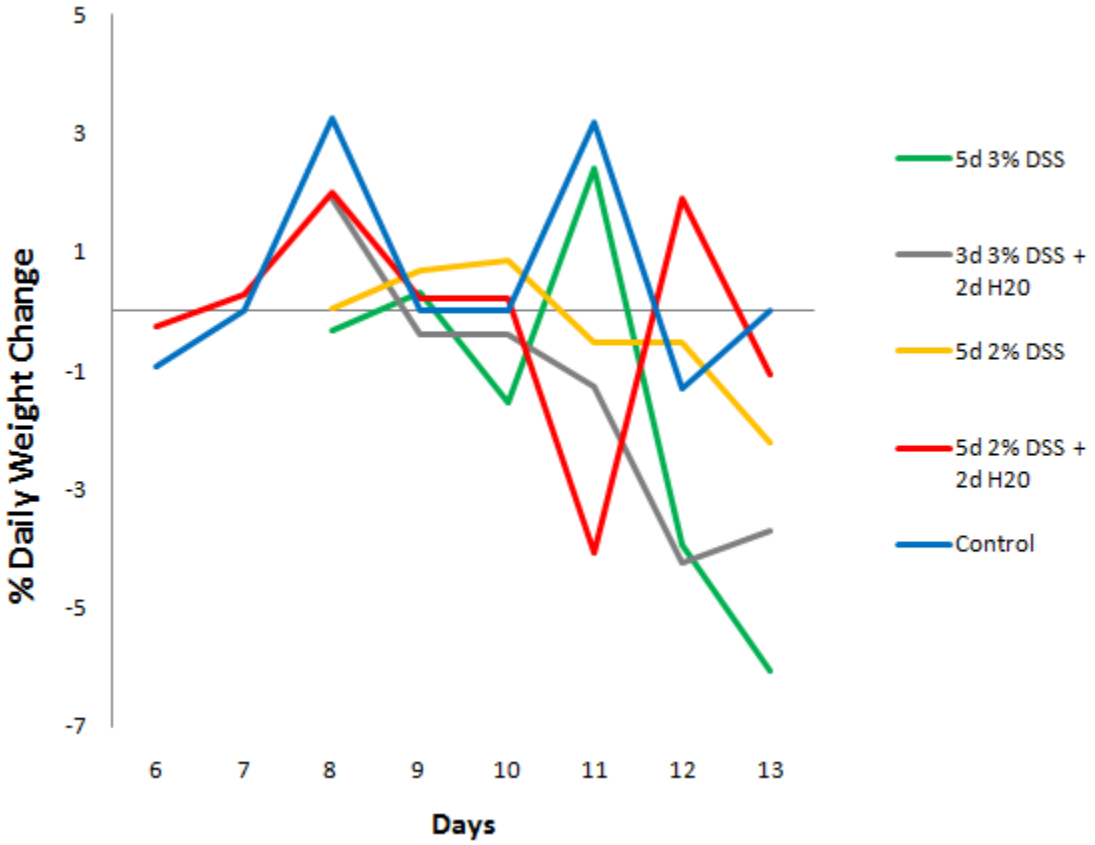
Mice which were given 2 to 3% DSS experienced mild to severe inflammation of the colon, which involved mild to severe weight loss (Figure 5 and Figure 6). Behavioral changes were also noticed in some mice, including lethargy and withdrawal. On day 5 of the 3% DSS treatment, mouse #6 was euthanized due to severe inflammation, including severely bloody and loose stools and >10% weight loss. Histopathology revealed that mice given 2% and 3% had severe inflammation of the distal and proximal colon, with no signs of recovery. Inflammation could be seen in H&E sections characterized by severe edema, infiltration by inflammatory cells,

shortened crypts and loss of goblet cells. Colon length measured at sacrifice was also not able to recover to match the control (Figure 7).

3.4 Conclusion to Pilot 1

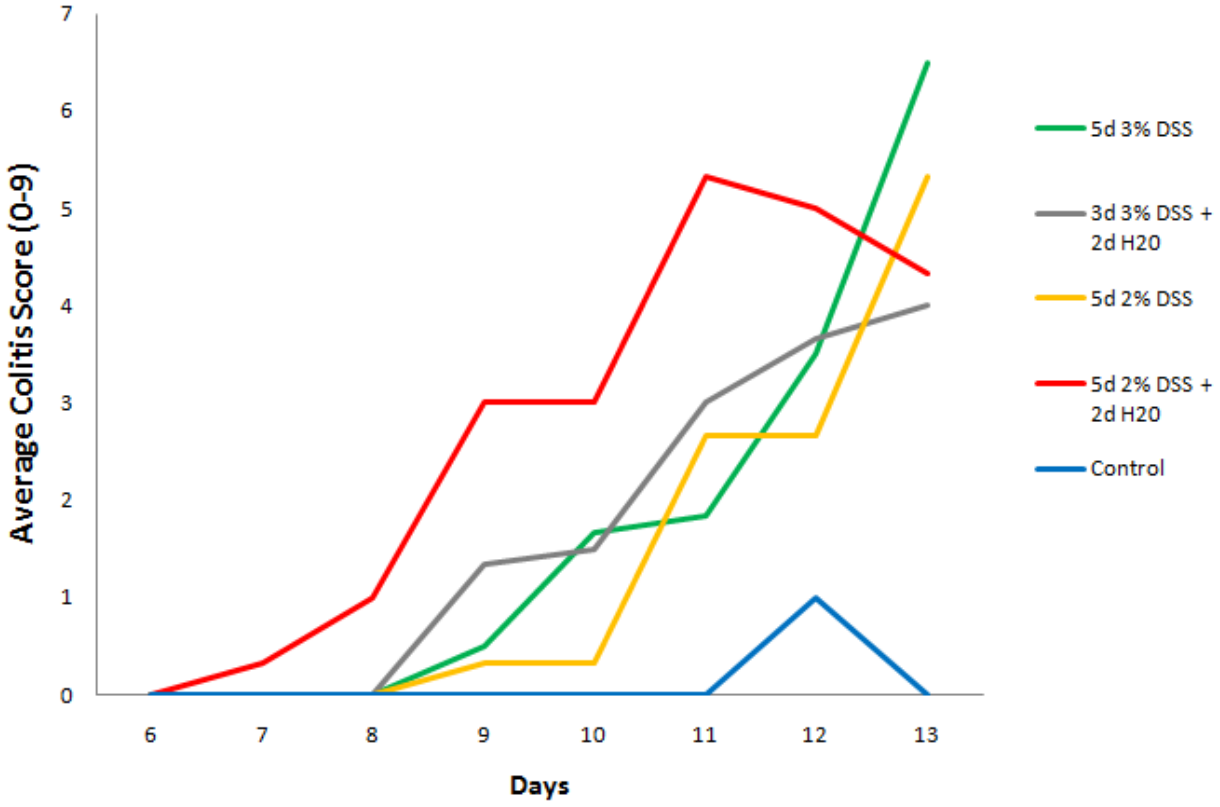
Based on these observations a second pilot study was devised using lower DSS doses to lessen the severity of inflammation, allowing the mice to recover from the insult with a longer study in mind. Since 2% and 3% DSS resulted in severe inflammation revealed by colon pathology we decided to lower the dose to 1% DSS for 5 days. We also decided to allow the mice a longer period to recover from the DSS treatment in order to see whether this would reduce the inflammation seen by observation and histopathology and eliminate mortality of any mice.

Figure 5. Percent weight change with DSS treatment (pilot study 1)[1]



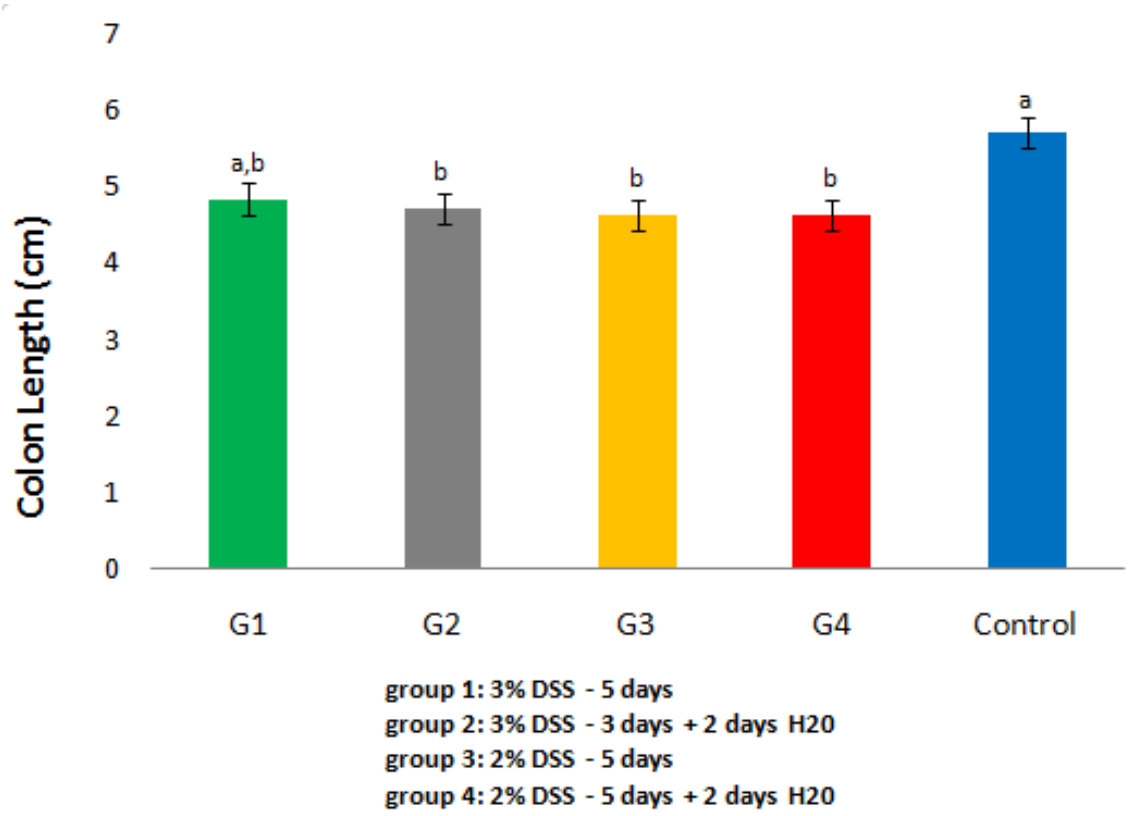
[1]Male, C57BL6 mice (n=6) weighing an average of 21.1 grams were provided Dextran Sulfate Sodium (DSS), or tap water, as their sole drinking water, as shown. Percent weight change was calculated from day to day during DSS treatment.

Figure 6. Disease Activity Index of mice during DSS treatment (pilot study 1)[1]



[1]Mice (male C57bl/6, n=6) were observed daily and given a colitis score based on weight loss, stool formation and fecal bleeding (table 3.2). Colitis scores were characterized as mild (1-4), moderate (5-7) or severe (8-9).

Figure 7. Effect of DSS on colon length of mice (pilot study 1)[1]



[1] Mice (male C57bl/6, n=6) were killed by cervical dislocation and colon was removed distal from the cecum to distal from the rectum and washed with 1.15% KCl. Colon length was measured from the cecal end to rectum. Mean $\pm$ SEM, one-way ANOVA, $p < 0.05$ using Fisher's LSD

3.5 Introduction: Inflammation Pilot Study 2

Objective: to establish the acute colitis model using DSS with a period of recovery so that mice will experience a mild to moderate colon inflammation that will not progress to mortality. The justification is to use this irritant along with the carcinogen, azoxymethane, in an inflammation enhanced model of colon cancer. Animal care, reagents, monitoring of overt signs and histopathology used in pilot 2 were identical to those used in pilot 1.

3.6 Methods

Mice were allowed 3 days acclimation before administering DSS in drinking water. Short recovery: 1% (w/v) DSS was dissolved in de-ionized, distilled water and given fresh daily to mice for 5 days (n=4) or 3 days with 2 days recovery on regular tap water (n=4). Long recovery: 1% (w/v) DSS was given to mice for 5 days with 7 days recovery (n=4) or 3 days with 9 days recovery (n=4). Control mice received regular drinking water without DSS and were killed immediately after acclimation on day 0 (n=4). At the end of the DSS treatments, mice were anesthetized using ketamine/xylazine; blood was drawn by cardiac puncture and mice were killed by cervical dislocation. Livers were immediately perfused with ice-cold isotonic KCl solution (1.15% w/v) and weighed. The colon was removed distal from the cecum to distal from the rectum, flushed with ice-cold KCl solution, measured, and the mucosal scraping was collected. All samples were snap frozen in liquid nitrogen and stored at -80˚C until further processing.

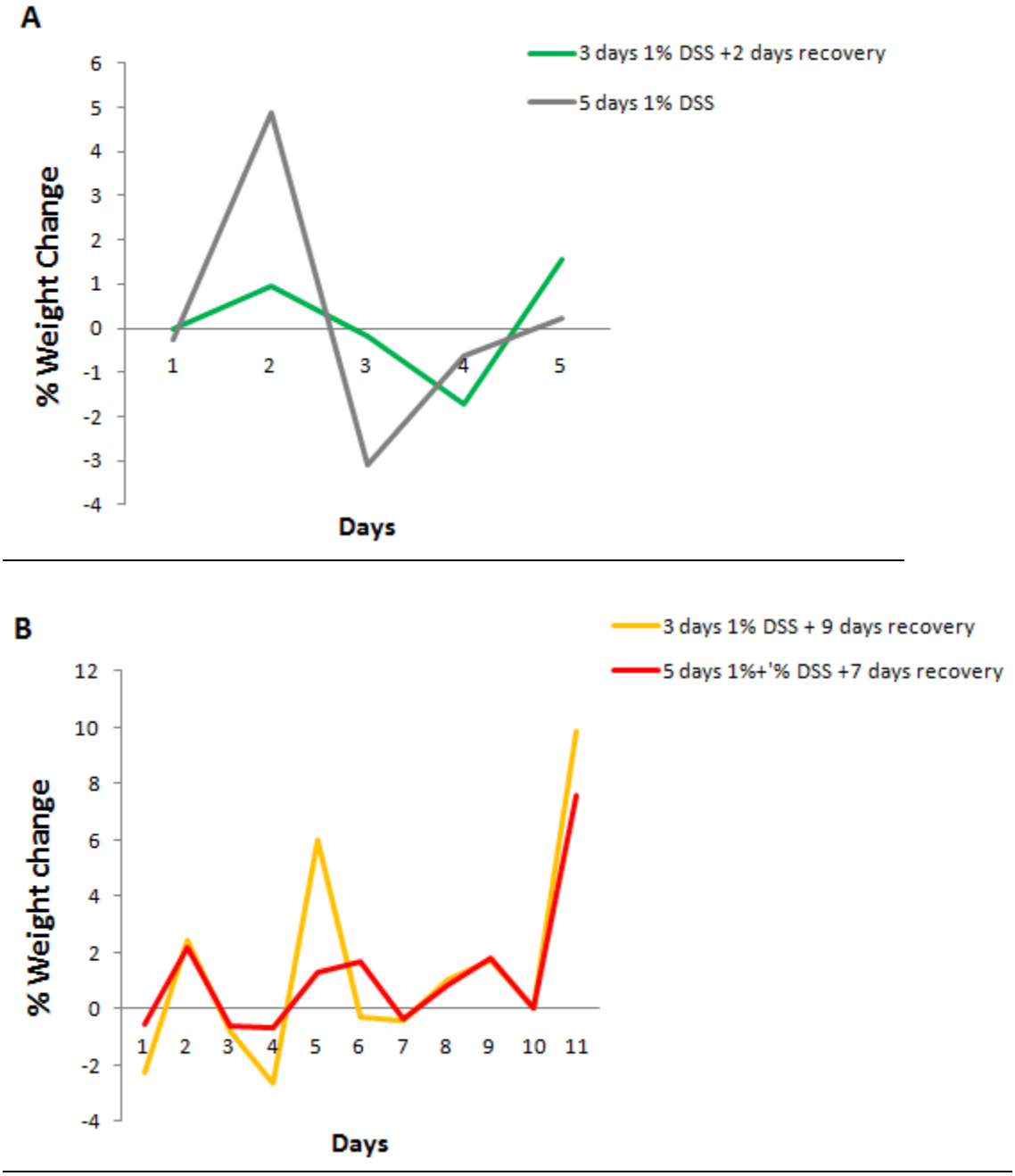
3.7 Results of Pilot 2

Mice which were given 1% DSS experienced mild colitis which involved mild to moderate weight loss (Figure 8 and Figure 9). Behavioral changes did not appear as great as the previous pilot study and no mice were required to be euthanized before the study was terminated. Histopathology revealed that some mice still experienced severe inflammation of the distal and proximal colon when given 1% DSS for 5 days. However with the recovery period, colon integrity was restored in many mice and only mild inflammation was observed. All mice experienced a mild degree of weight loss during DSS treatment, but weight was quickly regained once DSS treatment was stopped and the mice were allowed to recover. Colon length measured at sacrifice recovered sufficiently as to match control length in those groups given a period to recover (Figure 10).

3.8 Conclusion to pilot 2

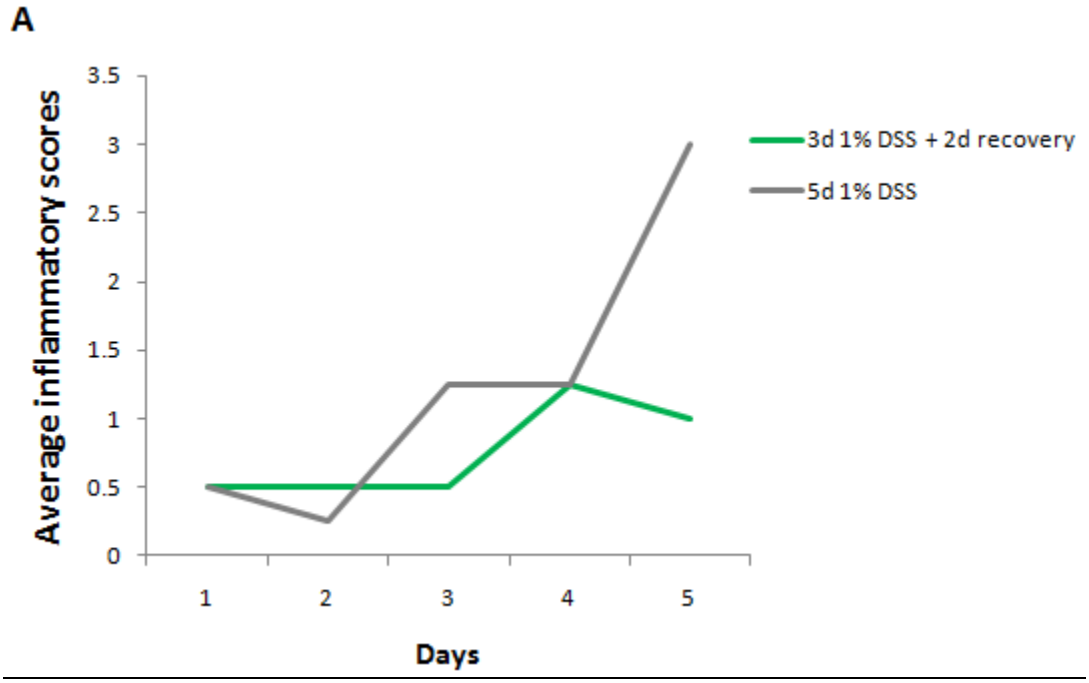
Mice were able to recover from the 1% dose of DSS given for 5 days. Based on these observations, a dose of 1% DSS was chosen for 7 days, after administering the carcinogen, azoxymethane. We chose 7 days to ensure that the mice were experiencing inflammation, in order to make the model a legitimate model of inflammation-enhanced colon cancer.

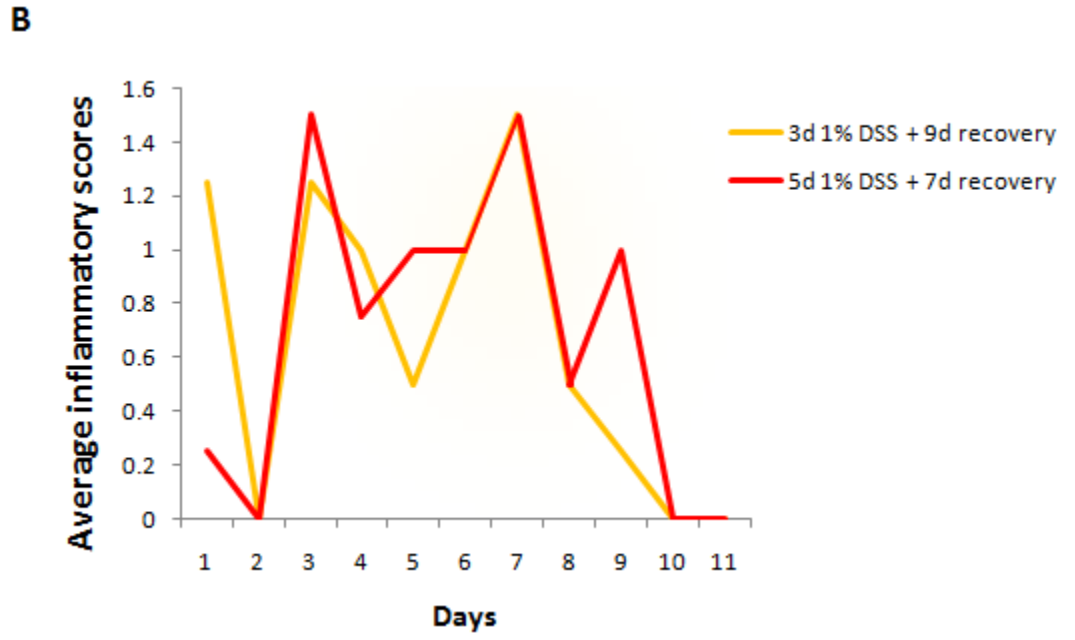
Figure 8. Percent weight change in DSS (pilot study 2)[1]



[1]Male, C57BL/6 mice were treated with DSS in their drinking water as follows; (A) short recovery: 1% DSS was dissolved in de-ionized, distilled water and given fresh daily to mice for 5 days (n=4) or 3 days with 2 days recovery on regular tap water (n=4). (B) Long recovery: 1% DSS was given to mice for 5 days with 7 days recovery (n=4) or 3 days with 9 days recovery (n=4). Body weight was measured daily and % weight change calculated for each day.

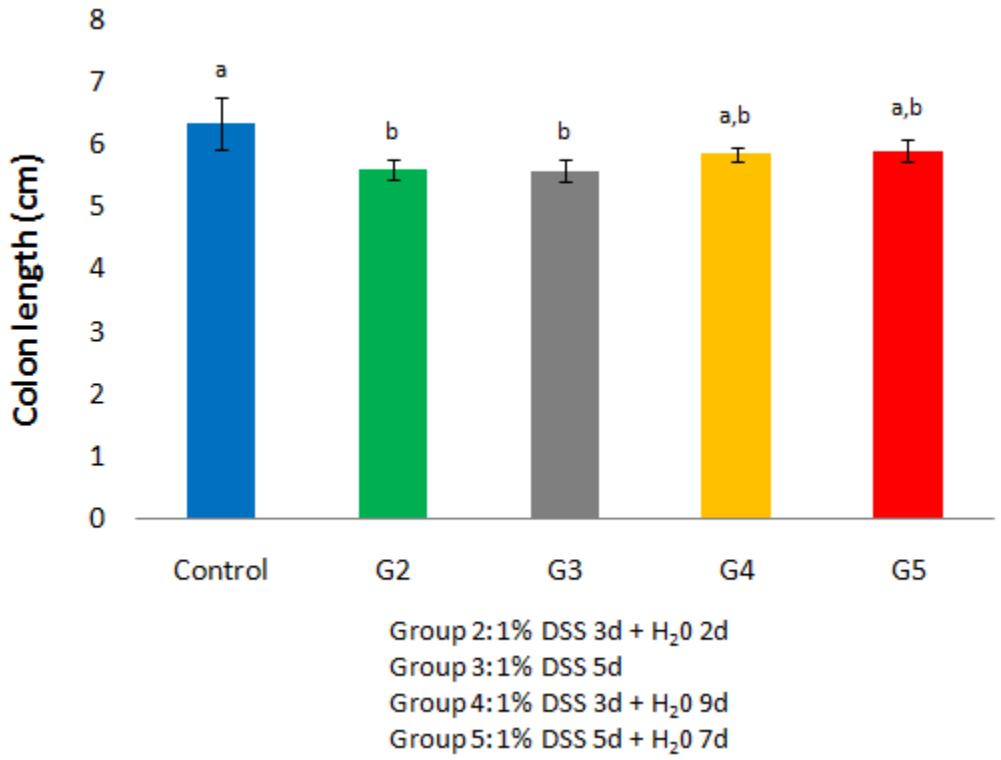
Figure 9. Disease Activity Index of mice during DSS treatment (pilot study 2)





[1] Male, C57BL/6 mice were treated with DSS in their drinking water as follows; (A) short recovery: 1% DSS was dissolved in de-ionized, distilled water and given fresh daily to mice for 5 days (n=4) or 3 days with 2 days recovery on regular tap water (n=4). (B) Long recovery: 1% DSS was given to mice for 5 days with 7 days recovery (n=4) or 3 days with 9 days recovery (n=4). Daily colitis scores were characterized as mild (1-4), moderate (5-7) or severe (8-9).

Figure 10. Effect of DSS treatment on colon length in mice (pilot study 2)



[1]Male, C57BL/6 mice were treated with DSS in their drinking water as follows; (A) short recovery: 1% DSS was dissolved in de-ionized, distilled water and given fresh daily to mice for 5 days (n=4) or 3 days with 2 days recovery on regular tap water (n=4). (B) Long recovery: 1% DSS was given to mice for 5 days with 7 days recovery (n=4) or 3 days with 9 days recovery (n=4). After being flushed with 1.15% KCl, colon length was measured distal from the cecum to distal from the rectum. Mean $\pm$ SEM, one-way ANOVA, $p < 0.05$ using Fisher's LSD.

CHAPTER 4
RAPPINI AND INFLAMMATION STUDY

4.1 Introduction

Broccoli (*Brassica oleracea* L.) diets have been shown to reverse inflammation caused by a number of agents, including inflammation of the colon due to DSS (104). Rappini (*Brassica rapa* L.) belongs to the same plant family as broccoli (*Brassicaceae*). Like broccoli, it is a rich source of aliphatic glucosinolates. However, the major glucosinolates in rapini are gluconapin and glucobrassicanapin (105). The isothiocyanate that is derived from gluconapin, 3-butenyl isothiocyanate, has been shown to inhibit the proliferation of HT29 colorectal cancer cells by disrupting the cell cycle, but there is no source indicating it has anti-inflammatory effects (106).The purpose of this study was to compare dietary broccoli and rappini (broccoli raab) for efficacy at reducing or mitigating acute inflammation induced by DSS in C57bl/6 mice. Our hypotheses were that mild to severe inflammation would be seen in mice receiving 1% DSS treatment for 7 days on the control AIN-93G diet. Colon sections of these mice would reveal the presence of numerous inflammatory cells, edema and shortened crypts. In mice receiving 1% DSS treatment and the 10% freeze-dried broccoli or rappini diets, inflammation would be ameliorated and colon sections would appear normal, with the absence of inflammatory cells, edema, and shortened crypts.

4.2 Methods

Animal Care:

Eight to ten week old male C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed individually in shoebox cages and maintained under 12-hour light/dark cycles at 22˚C and 60% humidity (University of Illinois, Urbana-Champaign).

All animals were allowed water and AIN-93G diet *ab libitum*. All animal care followed the protocol approved by the Institutional Animal Care and Use Committee at the University of Illinois, Urbana-Champaign, in accordance with NIH regulations.

Reagents:

All diet ingredients were bought from Harlan-Teklad laboratories (Madison, WI) and mixed following the Harlan-Teklad standard AIN93G composition (Table 3). Broccoli was grown, freeze-dried and powdered at the University of Illinois, Urbana, IL. Rappini was provided by D'Arrigo Bros. Co., Salinas, CA and freeze-dried at the University of Illinois, Urbana, IL. Broccoli and Rappini (10% w/w) were added to the diet (Table 6 and Table 7, respectively). Dextran sulfate sodium (M.W. 36,000-50,000 kDa) was purchased from MP Biomedicals, LLC (Solon, OH).

Experimental Design:

Mice were allowed 4 days acclimation (AIN93G *ad libitum*) before starting on treatment diets. After 4 days on treatment diets, 1% (w/v) DSS was dissolved in de-ionized, distilled water and given fresh daily to mice for 7 days, as their only source of water; regular tap water was given to control groups. Mice were divided into 6 groups (n=6 per group) and provided with 6 diets (table 4.1); I: Control (AIN93G), II: AIN93G+ DSS, III: 10% Broccoli diet, IV: 10% Broccoli diet + DSS, V: 10% Rappini diet, VI: 10% Rappini diet + DSS. Powdered diet was provided *ad libitum* and replaced fresh daily. At the end of the DSS treatments, mice were anesthetized using ketamine/xylazine as described in chapter 3. Blood was drawn by cardiac puncture and mice were killed by cervical dislocation. Livers were immediately perfused with

ice-cold KCl solution (1.15% w/v) and weighed. The colon was removed distal from the cecum to distal from the rectum, flushed with ice-cold KCl solution, the length measured, and the mucosa was scraped and collected. All samples were snap frozen in liquid nitrogen and stored at -80˚C until further processing. Monitoring of overt signs and histological methods were identical to the previous pilot studies. Mice were monitored using the Disease Activity Index described in Chapter 3 (Table 4).

Histopathological examination:

Severity of inflammation was determined by examination of H&E staining of colonic sections with the assistance of Dr. Matthew A. Wallig, Department of Veterinary Sciences (University of IL, Urbana, IL). Sections were determined to be normal or classified as mild, moderate or severe colitis. Sections were described as normal if they had no apparent mucosal/submucosal edema or presence of inflammatory cells (neutrophils, macrophages, etc) and if there were abundant goblet cells and the crypt epithelium was intact. Sections described as mild colitis had mild edema, minor shortening of the crypts and presence of low numbers of inflammatory cells. Sections described as moderate colitis had a greater presence of inflammatory cells, loss of goblet cells, more extensive edema, and irregular crypt variation and sometimes collapsed lamina propria. There were no sections characterized as severe colitis; however, these sections would have appeared to have marked edema, infiltration of numerous inflammatory cells, profound loss of goblet cells, severely shortened crypts, mitotic figures, inflamed muscularis and collapsed lamina propria.

Statistical Analysis:

Differences between groups were analyzed using Student's T-test and ANOVA followed by post hoc Tukey's test for multiple comparisons. $P \leq 0.05$ was considered significant. All data were analyzed using SAS statistical software (SAS Institute Inc., Cary, NC).

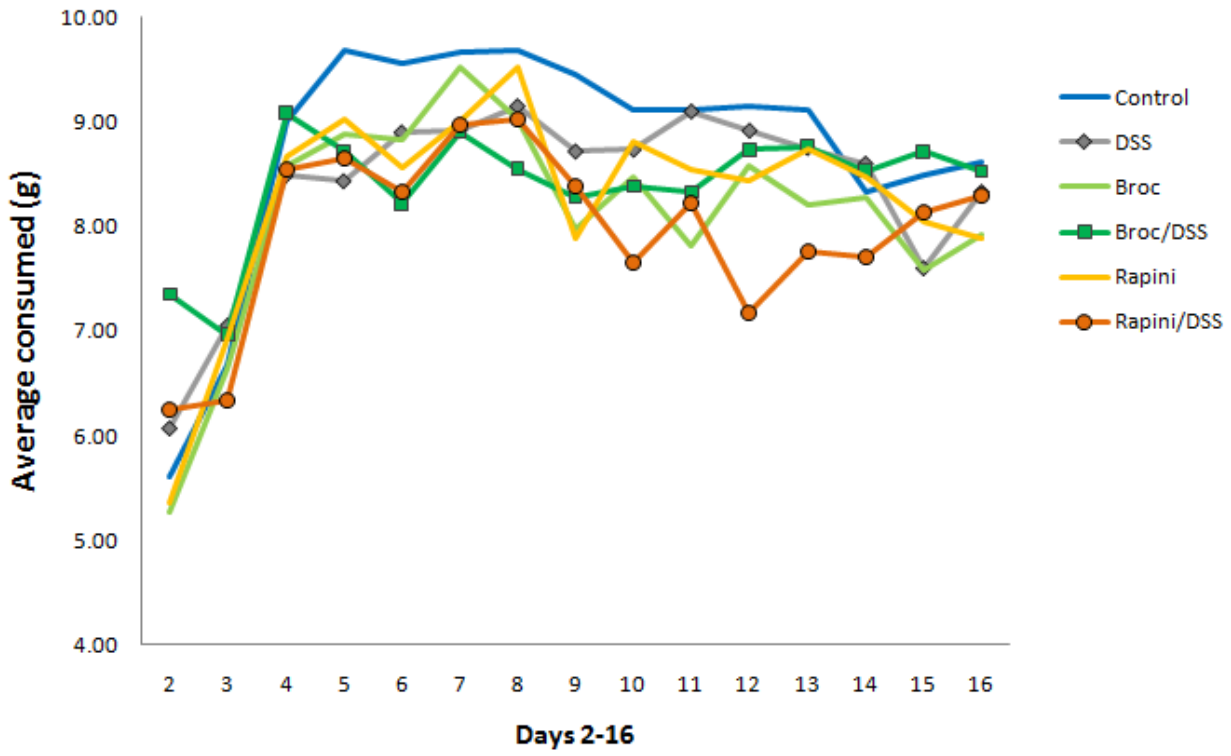
4.3 Results

Food intake in the rappini plus DSS group was lower while receiving DSS treatments, but recovered by day 16 when all groups had similar intake (Figure 11). Body weight within in the rappini groups was consistently lower throughout the study (Figure 12). Body weight did not differ significantly from group to group on the first day of the study. However, by the last day of the study the rappini plus DSS group had significantly lower body weight compared to all other groups; mice receiving rappini alone had significantly lower body weights compared to mice receiving the control diet. By the last day of the study, all groups had gained weight compared to the first day, except for the rappini plus DSS group ($p<0.05$). Colon length was significantly lower in the control (AIN-93G) DSS group when compared to the all other groups ($p<0.05$). Both the rappini plus DSS and broccoli plus DSS groups were able to maintain colon length, whereas the plus DSS group was 14% shorter than the control group receiving no DSS. Liver weights did not differ between groups ($p<0.05$). Histopathology showed that 66% of mice in the control plus DSS group had mild to moderate colitis; 33% of mice in the rappini plus DSS group had mild to moderate colitis; and 17% of mice in the broccoli plus DSS group had mild colitis (Table 5). These percentages do not correlate with the Disease Activity Index scores. Two mice within the broccoli plus DSS group exhibited traces of edema not considered serious enough to classify as mild colitis.

4.4 Conclusion

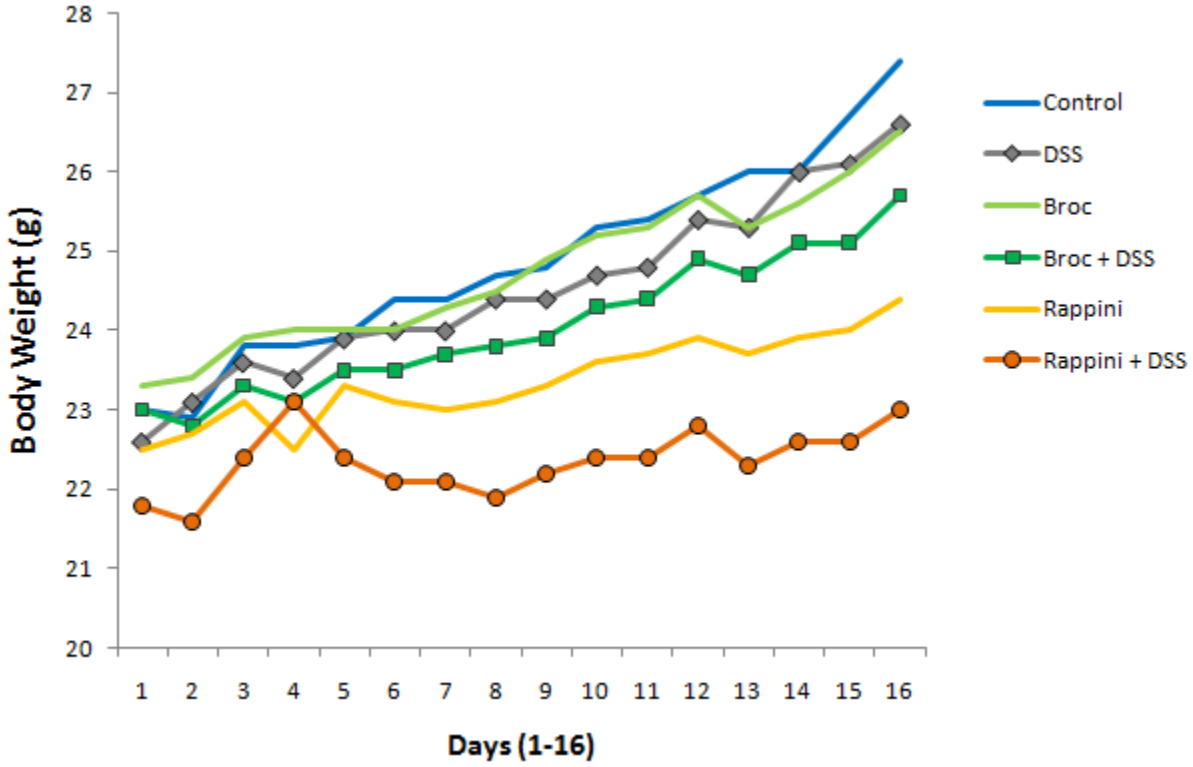
DSS (1%) caused mild to moderate colitis in the control group as revealed by histopathology. This was associated with shortening of the colon whereas dietary broccoli and rappini both protected against shortening of the colon from inflammation; rappini was not as effective at reducing the insult caused by DSS, as revealed by histopathology. Since broccoli seems to have a promising effect on reducing inflammation we decided to develop our inflammation model into a colon cancer model. Using azoxymethane, a chemical carcinogen, and DSS it is possible to induce colon cancer in mice that bears resemblance to that of human Ulcerative Colitis, with progression to colon cancer.

Figure 11. Food consumption in mice receiving dextran sulfate sodium for 7 days.[1]



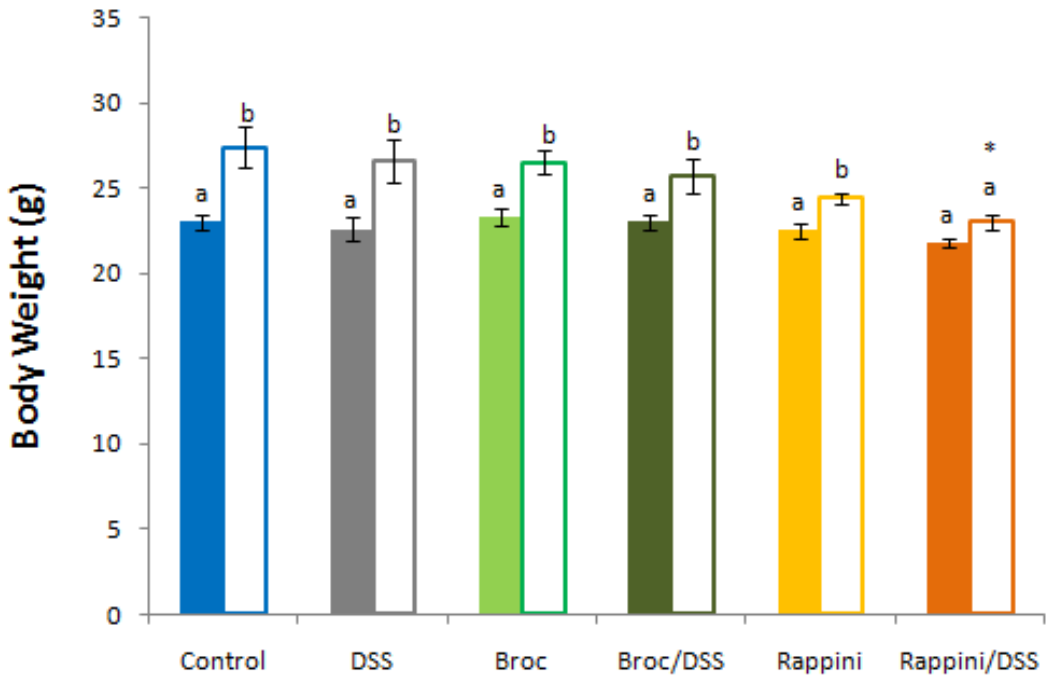
[1]Male, C57BL/6 mice were treated as described in methods, with AIN93G for days 1-4; treatment diets days 5-8 and treatment diets + DSS (1%) on days 9-16. Food consumption was recorded from days 2-16. Each line represents a daily average per group (n=6).

Figure 12. Mean body weight of mice receiving diets with and without dextran sulfate sodium in the drinking water.[1]



[1]Male, C57BL/6 mice were treated as described in methods; mice received AIN93G days 1-4; treatment diets days 5-8 and treatment diets + DSS (1%) days 9-16. Body weight of each mouse was recorded daily. Each line represents a daily average per group (n=6).

Figure 13. Mean body weight before and after DSS treatment.[1]

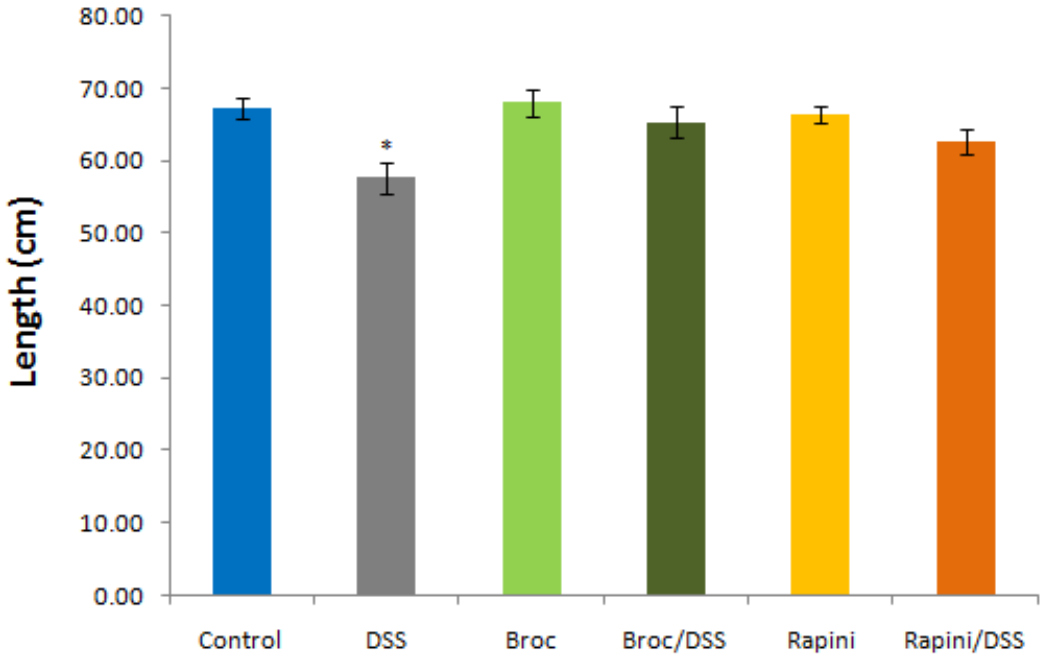


[1]Male, C57BL/6 mice were treated as described in methods: mice received AIN93G days 1-4; treatment diets days 5-8 and treatment diets + DSS (1%) days 9-16.

* Indicates significant difference between first day of DSS (day 7; filled bars) and last day (open bars). ($p < 0.05$) Data are means ± standard error. T-tests were performed separately to determine differences between control, control/dss, broc, broc/dss, rapini and rapini/dss.

Different letters indicate differences between DSS and no DSS within a dietary treatment group. Body weight is reported for day 0 and day 16 of study. Differences were found in all groups ($p < 0.05$) excluding the rappini/DSS group, which was not different from the beginning to the end of the study.

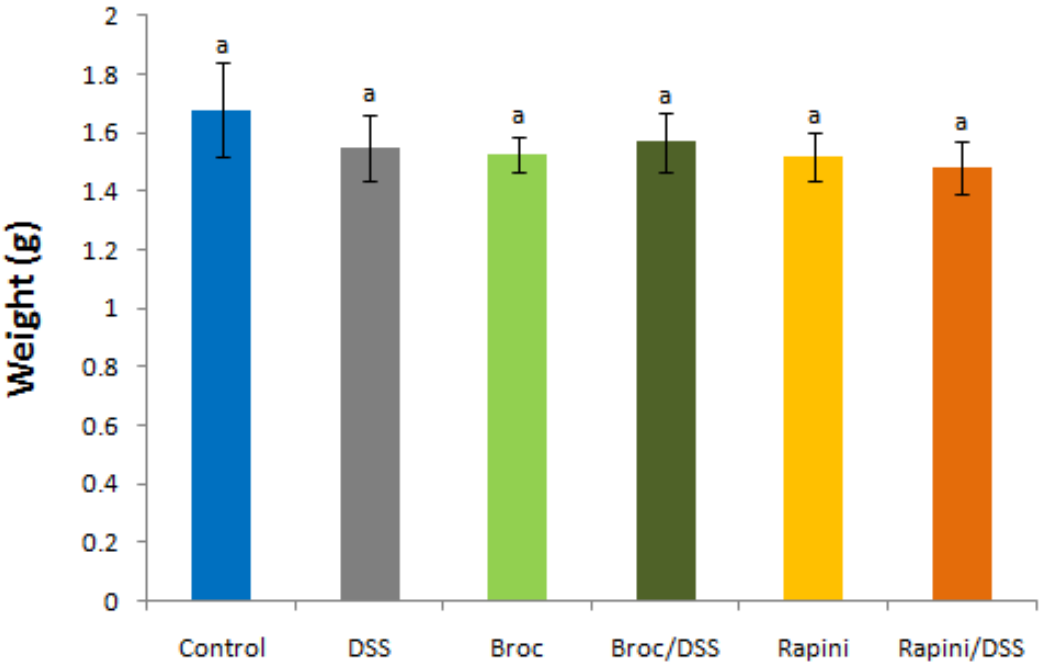
Figure 14. Effect of diet on DSS-induced decrease in colon length[1]



[1]Male, C57BL/6 mice were treated as described in methods: mice received AIN93G days 1-4; treatment diets days 5-8 and treatment diets + DSS (1%) days 9-16. Colon was removed and flushed with 1.15% KCl and measured distal from the cecal to distal from the rectum.

* Indicates a significant difference in colon length between groups. Data are mean ± standard error. Significant differences were determined by student's t-test ($p < 0.05$)

Figure 15. Effect of DSS and diet on liver weight[1]



[1]Male, C57BL/6 mice were treated as described in methods: mice received AIN93G days 1-4; treatment diets days 5-8 and treatment diets + DSS (1%) days 9-16. Livers were perfused with 1.15% KCl, removed, patted dry and then weighed. Different letters indicate significant differences. Data are mean ± standard error. All groups were found to be not different, using Tukey's test for multiple comparisons ($p < 0.05$).

Table 5. Level of colitis in DSS treated mice[1]

Treatment Group	Normal	Mild edema	Mild colitis	Moderate colitis	Severe colitis	% mice with colitis
AIN93G + DSS	2		3	1		66%
Broccoli + DSS	3	2	1			17%
Rappini + DSS	2	2		2		33%

[1]Male, C57BL/6 mice were treated as described in methods: mice received AIN93G days 1-4; treatment diets days 5-8 and treatment diets + DSS (1%) days 9-16. Severity of colitis was determined by histopathology.

Table 6. Broccoli diet composition

Formula (1 Kg diet)	AIN-93G (g/Kg)	10% Broccoli (g/Kg)
Broccoli	--	100
Casein	200.0	173.64
Celluose	50.0	25.70
Corn Starch	397.486	375.63
Maltodextrin	132.0	122.96
Sucrose	100.0	93.15
L-Cystine	3.0	3.0
Mineral Mix	35.0	26.87
Vitamin Mix	10.0	10.0
Choline Bitartrate	2.5	2.5
Soybean Oil	70	66.54

Broccoli, raw (source: USDA website)

Component	Value/100g	100g (dry wt=10.7g)
Water	89.3	
Energy	34 kcal	
Protein	2.82	26.36
Total fat	.37	3.46
Ash (total mineral)	.87	8.13
Carbohydrate (+fiber)	6.64	62.06
Fiber (total dietary)	2.6	24.3
Sugars (total)	1.7	15.89

Adjusted AIN-93G diet as follows:

Subtract protein from casein
Subtract total lipid from soybean oil
Subtract ash from mineral mix
Subtract starch portion of carbohydrate from corn starch
Subtract dietary fiber from cellulose
Subtract ratio of sugar from maltodextrin and sucrose (calculated below)
Starch portion = total carb – total fiber – total sugar

Broccoli starch potion = 62.06 – 24.3 – 15.89 = 21.87

Maltodextrin	**132**	**9.04 (=15.89*132/232)**
Sucrose	**100**	**6.85 (=15.89*100/232)**
Total sugar (broccoli)	**232**	**15.89**

Table 7. Rappini diet composition

Formula (1 Kg diet)	AIN-93G (g/Kg)	10% Rappini (g/Kg)
Rappini	--	100.00
Casein	200.0	157.45
Celluose	50.0	13.76
Corn Starch	397.486	400.58
Maltodextrin	132.0	129.1
Sucrose	100.0	97.8
L-Cystine	3.0	3.0
Mineral Mix	35.0	22.52
Vitamin Mix	10.0	10.0
Choline Bitartrate	2.5	2.5
Soybean Oil	70	63.42

Rappini (broccoli raab), raw (source: USDA website)

Component	Value/100g	100g (dry wt=7.45g)
Water	92.55	
Energy	22 kcal	
Protein	3.17	42.55
Total fat	.49	6.58
Ash (total mineral)	.93	12.48
Carbohydrate (+fiber)	2.85	38.26
Fiber (total dietary)	2.7	36.24
Sugars (total)	.38	5.10

Adjusted AIN-93G diet as follows:

Subtract protein from casein
Subtract total lipid from soybean oil
Subtract ash from mineral mix
Subtract starch portion of carbohydrate from corn starch
Subtract dietary fiber from cellulose
Subtract ratio of sugar from maltodextrin and sucrose (calculated below)
Starch portion = total carb – total fiber – total sugar

Rappini starch potion = 38.26 – 36.24 – 5.10 = -3.08

Maltodextrin	**132**	**2.90 (=5.10*132/232)**
Sucrose	**100**	**2.20 (=5.10*100/232)**
Total sugar (rappini)	**232**	**5.10**

CHAPTER 5
INFLAMMATION ENHANCED COLON CANCER STUDY

5.1 Introduction

Colorectal cancer is the second leading cause of death from cancer worldwide. Inflammatory Bowel Disease (IBD), which includes Ulcerative Colitis, increases the risk for developing colon cancer (107). Dietary prevention in the form of cruciferous vegetables exhibits an inhibitory effect on cancer development (108). Sulforaphane, which is the metabolic product of glucoraphanin, is an isothiocyanate present in broccoli. Sulforaphane has been shown to possess anti-carcinogenic and anti-inflammatory properties in animal models (13, 17). The purpose of this study was to determine the mechanisms by which sulforaphane and other components of broccoli provide protective effects against inflammation-induced cancer. These data are expected to enhance our capability to translate this knowledge to improving the health of people suffering from IBD or those at increased risk for colon cancer.

Similarly, the incidence of colon cancer in Korea is steadily on the rise (109). At the Korea Institute of Science and Technology (KIST), dietary compounds are being studied for the prevention of cancer. There are many plants that are unique to the mountains of Korea that could contain promising anti-cancer components, slowing or abolishing this increasing rate of colon cancer. In particular, one compound, Gymnasterkoreayne B (GKB), isolated from the extract of the indigenous Korean plant, *Gymnaster koraiensis*, has shown potential therapeutic efficacy, including upregulation of phase II detoxification enzymes when rats were fed this component in their diet for five days. The phase II enzymes are known to detoxify many carcinogens resulting in cancer prevention. Providing natural plant components such as GKB via the diet or through pharmaceutical intervention may be a way to reverse the trend of increasing colorectal cancer

incidence in South Korea by enhancing the natural physiological ways by which the body protects itself from carcinogens or promoters of carcinogenesis. Before such a product can be evaluated in humans, it is necessary to evaluate colon cancer prevention in rodents. This study evaluated colon cancer in the presence of an ethanol extract and an isolated component (GKB) from *Gymnaster koraiensis* gathered in Daegwallyeong, Korea.

5.2 Methods

Reagents:

All diet ingredients were bought from Harlan-Teklad laboratories (Madison, WI) and mixed following the Harlan-Teklad standard AIN93G protocol (Table 3). Dextran sulfate sodium (M.W. 36,000-50,000 kDa) was purchased from MP Biomedicals, LLC (Solon, OH). Azoxymethane was purchased from Sigma-Aldrich (St. Louis, MO) and stored at -20ºC until use. Goldenrod animal lancets were purchased from MEDIpoint Inc. (Mineola, NY). Isoflurane was purchased from The Department of Veterinary Medicine (University of Illinois). Proliferating Cell Nuclear Antigen (PCNA) antibodies and Cyclooxygenase 2 (COX-2) antibodies for immunohistochemistry were purchased from Abcam (Cambridge, MA). Serum collected at the end of the study was used to measure interleukin-6 concentrations by ELISA (BD Biosciences; San Jose, CA). Benzylisothiocyanate was purchased from LKT Laboratories Inc. St. Paul, MN.

Processing of dietary components:

Approximately fifty pounds (6 cases-14 bunches/case) of Ocean Mist broccoli heads (Castroville, CA) were purchased from Meijer grocery store in Urbana, IL. Broccoli was stored

at 4˚C while being processed on the same day of purchase. Broccoli stems were discarded, retaining about 2 inches of broccoli floret, measured from the top of the crown. The florets were snap-frozen using liquid nitrogen and immediately stored in plastic gallon bags at -80˚C. Frozen broccoli florets were then freeze-dried using the VirTis General Purpose Freeze Dryer, model 24DX24 (Gardiner, NY) located in the Edgar R. Madigan Laboratory at the University of Illinois, Urbana, IL. Freeze-dried broccoli was finely ground into a powder and stored at -20˚C until use. Air dried broccoli sprouts were provided by Caudill Seed Company (Louisville, KY) and stored at room temperature until use. An extract, which is a fraction from the Korean aster plant, *Gymnaster koraiensis*, and the isolated compound Gymasterkoreayne B, were provided by KIST (Gangneung, Republic of Korea) and stored at 4˚C until use.

Animal Care & Diets:

98 Male, C57BL/6 mice (6-8 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed individually in shoebox cages at the Institute for Genomic Biology animal facility and maintained under 12-hour light/dark cycles at 22˚C and 60% humidity (University of Illinois, Urbana-Champaign). All mice were allowed water and a powdered, semi-purified (AIN-93G) diet *ab libitum*. Mice were acclimated to the AIN-93G diet for 3 days (day -14 to day -12) and on diet containing the various treatments (Table 8) for 12 days (days -11 to -1) prior to administering carcinogen (AOM/DSS). They were randomly assigned to one of fourteen groups the first seven groups received AIN93G diet containing treatments as shown in table 5.1. The second seven groups received similar diets/treatments to the first group. However, they also received ip injection of azoxymethane (10 mg/kg) on day 0 and DSS (1%) in the drinking water on days 7-13 (Figure 16). Because of the significant amount

of fiber added as broccoli or broccoli sprouts, the AIN93G diets containing these were modified to balance for fiber (Table 6 and Table 16 for broccoli and broccoli sprouts respectively). Korean plant materials were added to the soybean oil within the diet at 250 and 500µmol/kg of the diet (Table 15).

Experimental Design:

On day 0, after the acclimation period, mice were randomly assigned to the groups shown in table 5.1. Groups 1-7 received tap water without DSS throughout the study (n=7 per group). Serum was collected at baseline (day 0) and twice during the study (days 35 and 70); Urine was collected on days 0 and 14 (at the completion of DSS treatment). Urine was collected by placing a mouse in a small box which allowed for air passage and contained a 96 well plate. Mice were frequently monitored and urine was collected over a 2 hour period from the plate. Blood was collected from mice by mandibular puncture using Goldenrod lancets after brief exposure to isoflurane, and allowed to clot overnight at 4ºC to collect serum. At day 105 (week 15 after AOM injection), mice were anesthetized using ketamine:xylazine (87mg/ml:13mg/ml respectively; 0.1mL/100g BW). Blood was collected by cardiac puncture and mice were killed by cervical dislocation. Livers were immediately perfused with ice-cold isotonic KCl solution (1.15% w/v) and weighed. The colon was removed distal from the cecum to distal from the anus, flushed with ice-cold 1.15% KCl, the length recorded, and the entire colon observed microscopically for visible lesions. Lesions were measured and placed in 10% formalin for 24 hours and stored in 80% ethanol at 4ºC until histopathological analysis. The mucosa remaining was scraped from the colon and stored in liquid nitrogen. Blood, colonic mucosa, and liver samples were snap frozen in liquid nitrogen and stored at -80˚C until further processing.

Monitoring of mouse health:

A Disease Activity Index (DAI) scoring system adapted from (58) was used to monitor changes in mouse health throughout the study (Table 4). Mice were monitored daily for changes in weight or health and a medical case record was started for any animal exhibiting changes. Mice placed on medical cases were observed daily for their individual symptoms (Table 9). Inflammatory scores were recorded and included percent weight loss, stool formation and presence of fecal blood. Mice experiencing severe inflammation were monitored by a veterinary technician within the facility and were euthanized early if they did not recover.

Histopathology:

Sections of colon and small intestine were fixed in 10% formalin for 24 hours. Sections were cut, washed and placed in 80% ethanol and stored at 4˚C until further processing at the Veterinary Diagnostic Laboratory (Urbana, IL). Fixed tissues were embedded in paraffin and 10 micrometer cross-sections were mounted and stained with hematoxylin and eosin. Slides were examined using an Olympus BX51 microscope, an Olympus DP70 three-chip camera and an Antec EN8900 series computer for storage of images. Tumors were characterized as malignant or benign by a trained pathologist, Matthew A. Wallig in the Department of Veterinary Medicine (University of IL, Urbana, IL).

Immunohistochemistry:

Colon sections were allowed to incubate for 1 hour in antibodies for Proliferating Cell Nuclear Antigen (PCNA) or Cyclooxygenase 2 (COX-2). Data were reported as percent

positively-stained nuclei. PCNA-IHC is a standard method used to measure cell proliferation within lesions (110), while staining for COX-2 is a measure of the severity of inflammation (111). Tumor fields were evaluated for hyperplasia, adenomas and adenocarcinomas. Normal fields evaluated included both distal and proximal sections from colons of carcinogen-treated mice. Normal fields within colons of carcinogen-treated mice were called such if they did not display adenomas, adenocarcinomas or hyperplasia. At least 500 cells were counted within each tumor field and at least 300 within each normal field.

$$\textbf{PCNA-labeling index (\%)} = \frac{\text{PCNA positive-stained nuclei}}{\text{(PCNA positive + PCNA negative nuclei)}} \times 100$$

$$\textbf{COX2-labeling index (\%)} = \frac{\text{COX2 positive-stained nuclei}}{\text{(COX2 positive + COX2 negative nuclei)}} \times 100$$

Phase I and II Detoxification Enzyme Activity

Activity of the hepatic phase I enzyme CYP1A was measured in the microsomal fraction as ethoxyresorufin *O*-deethylase (EROD) activity using the method of Paolini (112). The activity of the phase II enzyme NAD(P)H-quinone oxidoreductase 1 (NQO1) was measured in the cytosolic fraction according to the method of Prochaska and Santamaria (113). Methods for both detoxification enzyme measurements were modified slightly as previously reported (114, 115).

Analysis of sulforaphane within broccoli and broccoli sprouts:

For hydrolysis and SF analysis, freeze-dried broccoli powder and air-dried broccoli sprout powder was hydrolyzed by mixing 1:15 (w/v) with water and incubated without exogenous myrosinase at room temperature in the dark for 24 hours. Broccoli sprout hydrolysate was diluted 1:4 before extraction). Benzylisothiocyanate (0.5 mg/mL) was added as internal standard and the sample extracted into dichloromethane (1:2) and measured by gas chromatographic analysis, as previously described (116). Sulforaphane content for broccoli and broccoli sprouts was 0.72μmol/g DW and 7.66μmol/g DW, respectively.

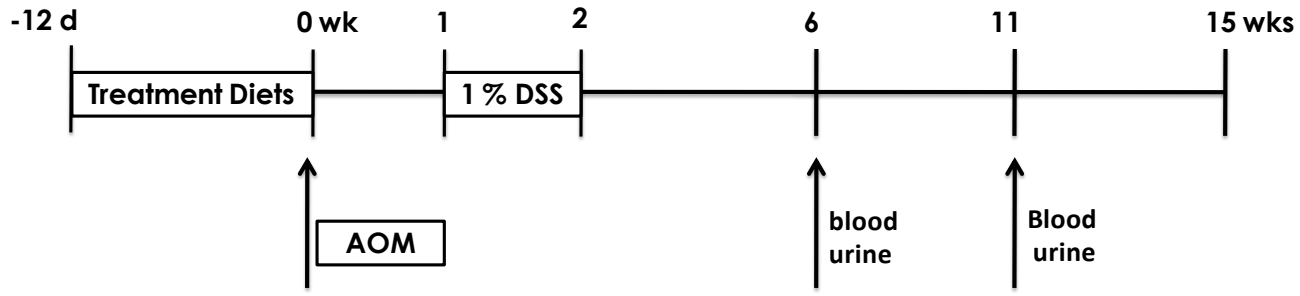
Statistical Analysis:

Data were analyzed using SAS statistical software (SAS Institute Inc., Cary, NC). Data were compared among treatments using one-way ANOVA with Fisher's LSD post-hoc for multiple comparisons, and compared between treatment and control (AIN93G) by Student's t-test and the Mann-Whitney non-parametric test. Values are means ± SEM, $p \leq 0.05$ was considered significant.

Table 8. Experimental diets

Group	*Treatment*
1	Blank Control (AIN93G diet)
2	10% Broccoli
3	5% Broccoli Sprouts
4	Low Gymnasterkoreayne B (GKB), 250 µmol/kg
5	High GKB, 500 µmol/kg
6	Low Gymnaster Extract (GE), 250 mg/kg
7	High GE, 500 mg/kg
8	Negative Control (AIN93G) + AOM/DSS
9	10% Broccoli + AOM/DSS
10	5% Broccoli sprouts + AOM/DSS
11	Low GKB, 250 µmol/kg + AOM/DSS
12	High GKB, 500 µmol/kg + AOM/DSS
13	Low GE, 250 mg/kg + AOM/DSS
14	High GE, 500 mg/kg + AOM/DSS

Figure 16. Experimental timeline



5.3 Results

We had expected to complete the study at week 20 and harvest samples. However, tumors grew faster than we anticipated and we ended the study at week 15. Most mice looked healthy at all times as measured by the Disease Activity Index (DAI) scores and the weekly

comments from the veterinary technician. All mice showing adverse signs and thus necessitating a medical case report were those that received AOM/DSS (Table 9). Six medical cases were culled early due to unlikely recovery from severe inflammation (Table 10). By week 15, the DAI scores revealed that mice receiving the broccoli and high Gymnaster Extract (GE) diets experienced fewer symptoms associated with inflammation, from that seen in mice receiving AOM/DSS. Those receiving the high GE diet exhibited the least inflammation (Figure 17).

Body weight and food intake:

Figure 18 shows food intake for the first 2 weeks of the study. Food intake dropped in all treatment groups after AOM administration (day 0), but recovered during (day 7-13) and was fully recovered by day 14, after DSS treatment. In mice receiving the herbal fraction (GE) and the isolated compound (GKB) but no carcinogen, there were no significant differences in food intake and body weight compared to control during the entire study. In mice receiving the herbal fraction and compound and AOM/DSS there was also no significant impact on food intake or weight gain. Food intake was not significantly affected by dietary treatment of broccoli. Figure 19 shows weekly body weights between treatment groups. Body weight did not differ between treatment groups until the final week, when weight of mice receiving AOM/DSS and the broccoli sprouts diet was significantly reduced compared to AOM-free mice receiving sprouts (Figure 20).

Effect of dietary and AOM/DSS treatments on colon length and liver weight:

Typically, the colon is shortened in chronic inflammation (60). Mice receiving the standard AIN93G diet and AOM/DSS had significantly shorter colons (Figure 21). With the

exception of the high dose A164a diet (500μmol/kg diet), mice receiving the KIST herbal treatments did not experience significant shortening of the colon. The broccoli and broccoli sprouts diets were comparable in that mice receiving these diets did not exhibit significant signs of colon shortening. Changes in liver weight may indicate potential toxicity resulting from exposure to dietary components or other chemicals (117). Mice receiving the sprouts, low compound and low GKB diets along with AOM/DSS had a significant reduction in liver weight as compared to their AOM-free counterparts (Figure 22).

Incidence and multiplicity of colon tumors:

Colons were evaluated for total number of tumors (Table 11), including adenomas (AD), which are considered pre-cancerous and adenocarcinomas (ADC) which are cancerous (118). The average number of adenocarcinomas was significantly reduced by two dietary treatments: high fraction diet (Hi-GE) and the 10% broccoli diet. Both of these diets decreased malignant adenocarcinomas by 90% when compared to AOM/DSS alone. None of the other diets caused a significant decrease in the number of adenocarcinomas, compared to mice receiving AIN93G plus AOM/DSS. The average number of adenomas per mouse was not significantly decreased by any of the treatments. The high dose of GE and the broccoli diets proved to have the most substantial effect overall on reducing tumor multiplicity.

Dietary treatment effects on proliferation and inflammation in normal and tumor tissue:

Hyperproliferation of cells is a critical feature in the initiation and progression of colon cancer (119). Cell proliferation has been shown to increase in the DSS/AOM mouse model during and after administration of the carcinogen (120). Results from the immunostaining for

Proliferating Cell Nuclear Antigen appear in Table 12. Both the high GKB and high GE treatment diets (500 μmol/kg diet) significantly decreased cell proliferation in tumor tissue. Proliferation was not altered in normal tissue surrounding the tumor tissue in mice receiving the high GKB and high GE diets. Cell proliferation was significantly reduced in tumor tissue of mice treated with the 10% broccoli diet. The 5% sprouts diet caused a slight reduction, but this was not found to be different from the AIN93G group. Similar to the lack of effect the Gymnaster diets on cell proliferation in surrounding normal tissue, neither 10% broccoli nor 5% broccoli sprouts impacted proliferation rates in normal tissue surrounding tumor tissue or tissue from mice receiving the AIN93G diet. Within treatment groups, proliferation in tumor and normal tissue was found to be significantly different, except in the high GKB treatment group.

Epidemiological and clinical studies demonstrate that anti-inflammatory drugs, such as aspirin, are effective in reducing the progression of colon cancer. This efficacy is attributed to the inhibition of COX-2 (121). Several different COX-2 inhibitors have been shown to significantly suppress tumorigenesis in the DSS/AOM mouse model (122). In this study, we show a significant reduction in expression of COX-2 in tumor tissue from mice given either the high GKB or high GE diets (Table 13). There was no significant difference in COX-2 expression between treatment groups within normal tissue. Interestingly, in mice given the high GE diet, the expression of COX-2 within tumor tissue was found to be significantly reduced when compared to expression in normal tissue. Similar to the Gymnaster diets, broccoli and broccoli sprouts diets were found to reduce induction of inflammation significantly, measured as COX-2 expression, within tumor tissue when compared to tissue from mice on control, AIN93G diet. No significant differences in expression were found between dietary groups in normal tissue. COX-2 expression within tumor and normal tissue fields of broccoli dietary groups was not statistically different.

Interleukin 6 (IL-6) is expressed in response to inflammation and is thought to trigger COX-2 expression and resulting prostaglandin E2 synthesis, leading to promotion of tumor cell differentiation and growth (123-125). Tables 14 shows the results from the Enzyme-Linked ImmunoSorbent Assay for IL-6 carried out on serum collected at cull. The high GKB (500µmol/kg diet) and low GKB (250µmol/kg diet) diets slightly decreased IL-6 in the serum of AOM mice, but this decrease was not significant. However, the low GE (250µmol/kg diet) and high GE (500µmol/kg diet) diets significantly reduced serum IL-6 when compared to AIN93G in mice given AOM/DSS. There were no significant differences found in IL-6 levels between any dietary groups within the control (AOM-free) mice. Serum IL-6 levels in control and AOM/DSS-treated mice were not found to differ for either the high GKB or low GKB diets. The 10% broccoli and 5% broccoli sprouts diets reduced levels of IL-6 in the serum of AOM treated mice. Surprisingly, among the control (no AOM/DSS) mice, serum levels of IL-6 in mice treated with broccoli and broccoli sprouts showed a significant increase when compared to mice given the AIN93G diet and no AOM/DSS. Levels of IL-6 were not statistically different between control and AOM/DSS-treated mice within broccoli and broccoli sprouts treated mice.

Dietary effect on hepatic detoxification enzyme levels:

Phase II enzyme induction can protect against cancer initiation by detoxifying reactive oxygen and nitrogen species and neutralizing electrophiles and radicals (126). In particular, induction of the phase II enzyme NAD(P)H:quinone reductase (NQO1) has been offered as a possible mechanism by which dietary bioactive components can suppress the initiation of colon cancer (127, 128). Figure 23 shows the effects of dietary treatments on NQO1 induction in the liver of control mice and AOM/DSS-treated mice. Interestingly, mice treated with AOM/DSS

plus the high GE diet significantly increased NQO1 activity over the control, AIN93G-fed mice. This correlates with the increases in NQO1 activity found by KIST scientists in their *in vitro* work using these same compounds (1). No other notable differences were found in NQO1 activity between dietary treatments. Figure 23b shows the effects of broccoli and broccoli sprouts on NQO1 enzyme induction in the liver. 10% broccoli had no significant effect on NQO1 activity, however the 5% broccoli sprouts diet and AOM/DSS-treated mice had significantly higher NQO1 activity in liver tissue, when compared to livers from mice receiving the control AIN93G diet.

The cytochrome P450 enzyme family plays a major role in drug and xenobiotic metabolism and is involved in carcinogen activation (91). Cytochrome P450 1A (CYP1A) is known to be induced by some dietary compounds, measured as an increase in EROD activity. Figure 24 shows the results of the EROD assay in mice receiving the dietary treatments. Mice treated with AOM/DSS and high GE had significantly higher EROD activity when compared to AOM/DSS-treated mice receiving the AIN93G diet. No other significant differences were found between treatment groups; however, for mice on most experimental diets, hepatic EROD activity was elevated when compared to livers of mice on the basal, AIN93G diet. Figure 24b shows EROD activity in livers of mice given broccoli or broccoli sprouts. Livers from mice not receiving AOM/DSS, but given 10% broccoli had significantly higher EROD activity when compared to livers from saline or AOM/DSS-treated mice receiving AIN93G. Hepatic EROD activity in mice fed broccoli sprouts was not statistically different from that of AIN93G-treated mice.

5.4 Discussion

In this study the extract from *Gymnaster koraiensis* was found to have potential as a chemopreventative agent against inflammation-enhanced colon cancer. The high dose of GE, but not pure GKB, caused a significant 90% drop in ADC per mouse, with no change in AD per mouse, possibly indicating decreased progression from AD to ADC. Mice given the high GE diet in addition to AOM/DSS not only exhibited significantly reduced tumor multiplicity, but had significantly lower DAI scores, normalized colon length, and decreased colonic epithelial COX-2, PCNA labeling and serum IL-6 levels in comparison to mice receiving AOM/DSS and the control diet. Significant reduction of ADC was not seen in mice receiving the low GE diet plus AOM/DSS; however, this diet was just as effective as the high GE diet at reducing inflammation measured by serum IL-6 levels, COX-2 and PCNA labeling indices. In contrast, whereas isolated GKB may also possess some anti-inflammatory effects as shown by decreased COX-2 and PCNA labeling, it was not as effective as the high GE diet at suppressing DAI scores or at maintaining colon length, and was less able to decrease tumor multiplicity compared to the GE.

In an earlier study, NQO1 was seen to increase with GKB treatment of hepatocytes in culture and in livers of rats given GKB for 5 days [2]. Although there was no significant increase in NQO1 over control caused by GKB in the current study, it is possible that mice may adapt to chronic exposure to GKB, such that any change in NQO1 activity seen in the first week of the study would diminish by week 15. In contrast, GE caused a persistent induction of NQO1. Changes in detoxification enzymes, such as NQO1, frequently indicate lowered tumor incidence and multiplicity. In this study, the persistent enhancement of NQO1 may be part of the reason that GE but not GKB significantly reduced tumor multiplicity in mice.

The xenobiotic response element (XRE) is involved in the transcriptional upregulation of CYP1A through the aryl hydrocarbon receptor pathway. The XRE is known to be upregulated in response to procarcinogens, such as polycyclic aromatic hydrocarbons and other environmental pollutants. GKB did not significantly induce EROD activity. This data is consistent with earlier findings *in vitro*, that the lack of the XRE, required for EROD induction, did not impact the effect of GKB [2]. However, GE caused an increase in EROD. These results indicate that GE may contain a variety of components capable of upregulating either phase I or phase II detoxification enzymes. This is similar to our chemoprevention studies using broccoli and broccoli sprouts as a dietary treatment, where sprouts only provide aliphatic isothiocyanates and induced NQO1, yet mature broccoli, known to provide a lower level of isothiocyanates, but to also provide indole metabolites, only induced the phase I enzyme EROD.

For this study, the dose of GE was normalized to the concentration of GKB. Yet the extract was a more effective anti-inflammatory and anti-tumor agent than purified GKB. Several compounds found in *Gymnaster koraiensis* are structurally related to GKB, all of which might add to the anti-cancer impact of the extract. Gymnaster koreayne A-F were isolated from GK and tested for cytotoxicity in the L1210 mouse leukemia cell line [24]. While not the most potent inducer of tumor cell death in those *in vitro* studies, GKB caused significant cytotoxicity (ED_{50} value of 3.3µg/mL); ED_{50} values for other components, found in less quantity, ranged from 2.1 to 9.6µg/mL [24]. Recently, several other compounds isolated from GK were tested for cytotoxicity in four different human tumor cell lines. Although purified GKB was not found to have a significant effect in that study, there were three other compounds which had a moderately cytotoxic effect in the cell lines tested, including a colon cancer cell line, HCT15 [1]. Interestingly all three of those preparations contained the flavonoid apigenin. Apigenin is anti-

carcinogenic flavone which can be found in carrots, celery and cilantro. Apigenin has recently been shown to significantly reduce high multiplicity, aberrant crypt foci formation in AOM-treated rats, decreasing proliferation and increasing apoptosis (1 mg/kg diet) [25]. However, apigenin is found in the ethyl acetate fraction of GK, whereas we treated mice with the hexane fraction, which is devoid of apigenin. The hexane fraction contains other minor components, including Gymnasterkoreaynes B, C, E, and 2,9,16-heptadecatrien-4,6-dyne-8-ol, one or more of which may synergize with GKB to provide the protection against tumorigenesis we saw from GE. As mentioned earlier, Gymnasterkoreaynes B, C and 2,9,16-heptadecatrien-4,6-dyne-8-ol have been found to provide substantial cytotoxicity against L1210 tumor cells with ED50 values ranging from 0.12-3.3 μg/mL (129, 130). These data suggest that future studies should be directed toward a comparison of the whole herb with a combination of polyacetylenes and flavonoids found within the whole herb, *Gymnaster koraiensis.*

The 10% broccoli diet was found to have potential as a chemopreventive agent against inflammation-enhanced colon cancer. The 10% broccoli diet, but not the 5% broccoli sprouts diet, caused a significant 90% drop in ADC per mouse, with no change in AD per mouse, possibly indicating decreased progression from AD to ADC. Mice given the 10% broccoli diet in addition to AOM/DSS not only exhibited significantly reduced tumor multiplicity, but had significantly lower DAI scores, normalized colon length and liver weight, and significantly reduced colonic epithelial COX-2, PCNA labeling and serum IL-6 levels in comparison to mice receiving AOM/DSS and the control diet. Similar to the situation between GE and GKB, the 5% broccoli sprouts diet possessed some anti-inflammatory effects as shown by reduced serum IL-6 and COX-2, but it was not as effective at suppressing DAI scores or maintaining body weight

and liver weight and was less able to decrease tumor multiplicity compared to the 10% broccoli diet.

Broccoli is a known inducer of EROD activity, while broccoli sprouts are known to induce NQO1 activity in the liver (131-135). In keeping with these known observations, there was no significant increase in NQO1 over control caused by 10% broccoli in the current study, while NQO1 activity was enhanced by broccoli sprouts. In contrast, no change over control was seen for EROD activity in broccoli sprouts treated mice; however, 10% broccoli treated mice had enhanced EROD activity in the liver over control mice. The role of CYP1A-dependent EROD induction in reduction of tumor incidence is controversial, with many scientists concerned that induction of CYP1A may enhance incidence of cancer, since many polycyclic carcinogens, such as dimethylbenzanthracene, are activated by CYP1A. This is not the case with azoxymethane, which is metabolized in the liver to its active carcinogenic form, methylazoxymethane, by Cytochrome P450 2E1 (136). Thus, enhanced EROD activity may be part of the reason broccoli was able to provide more protection against colon tumor advancement than broccoli sprouts in mice.

Table 9. Summary of Medical Cases

ID	Treatment	Weight loss	Stool formation	Fecal bleeding	Other
50	AIN-93G	1-5%	Moderate loose stool	Moderate bleeding	
18		1-5%	Mild loose stool	Moderate to gross bleeding	
97	Broccoli	1-5%	Normal	Mild bleeding	
87		None			Mass on abdomen
74	Sprouts	1-5%	Normal	Moderate bleeding	
41		5-10%	Mild loose stool	Moderate bleeding	
53		None	Normal	Moderate to gross bleeding	
15	Hi-GKB*	None	Normal	Gross bleeding (recovered after 1 wk)	
69		5-10%	Normal	Moderate to gross bleeding	
28	Lo-GKB**	None	Normal	Moderate bleeding	
16		None	Normal	Moderate to gross bleeding	
60		None	Normal	Mild bleeding	
42	Hi-GE[1]	None	Normal	Mild to moderate bleeding	
1		None	Mild loose stool	Moderate bleeding	
12	Lo-GE[2]	None	Normal	Mild bleeding	
7		None			Mass on abdomen
44		1-5%	Normal	Gross bleeding (recovered after 1 wk)	

*Containing 500 umol/kg diet A164a

**Containing 250 umol/kg diet A164a

[1]Containing 500 umol/kg diet DFR-11

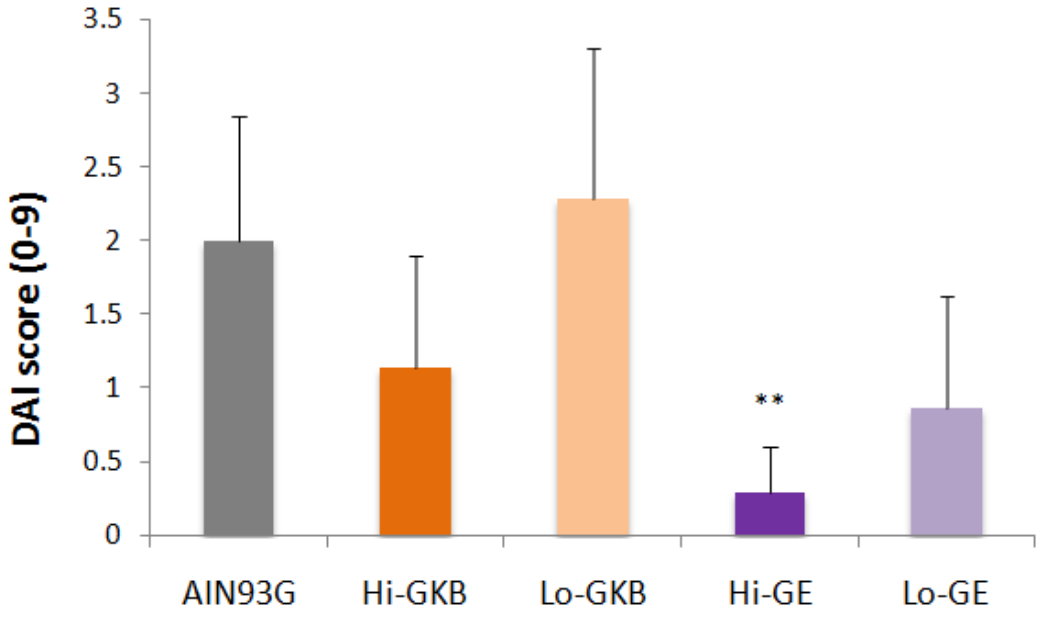
[2]Containing 250 umol/kg diet DFR-11

Table 10. Mice culled early for medical causes

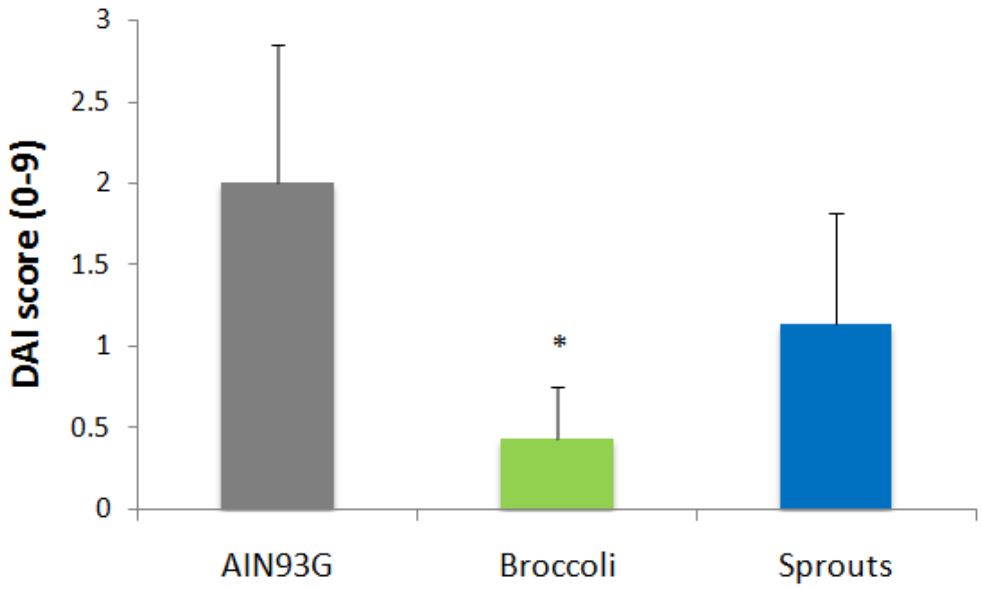
ID	Treatment	Weight loss	Stool formation	Fecal bleeding	Other
63	AIN-93G	1-5%	Moderate loose stool	Mild-gross bleeding	Prolapsed rectum
29	Sprouts	5-10%	Mild-moderate loose stool	Gross bleeding	Necrotic tissue/prolapsed rectum
34	Hi-GKB	1-5%	Mild loose stool	Gross bleeding	Necrotic tissue on rectum
65	Lo-GKB	5-10%	Mild-moderate loose stool	Gross bleeding	Dehydration, extreme lethargy
13	Hi-GE	5-10%	Mild-moderate loose stool	Gross bleeding	Prolapsed rectum
11	Lo-GE	1-5%	Moderate loose stool	Moderate bleeding	Ulcerated skin/prolapsed rectum

Figure 17. Comparison of Disease Activity Index (DAI) scores between AOM/DS-treatment groups during the week of sacrifice[1]

A. Average DAI scores of mice receiving control or Korean herbal diets



B. Average DAI scores of mice receiving control or broccoli diets



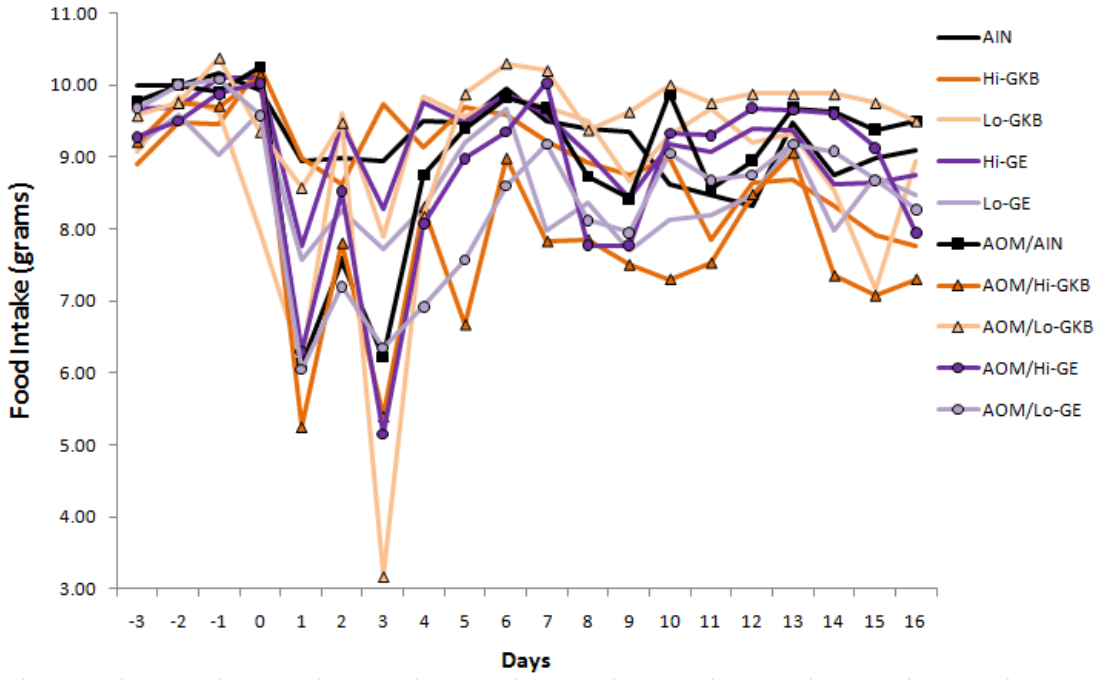
[1] Mice receiving different diets but no AOM/DSS all had DAIs of zero (data not shown).
Means ± SEM, $p < 0.05$ using Mann-Whitney test against the AIN-93G group

* Significantly different from AIN-93G ($p < 0.07$)

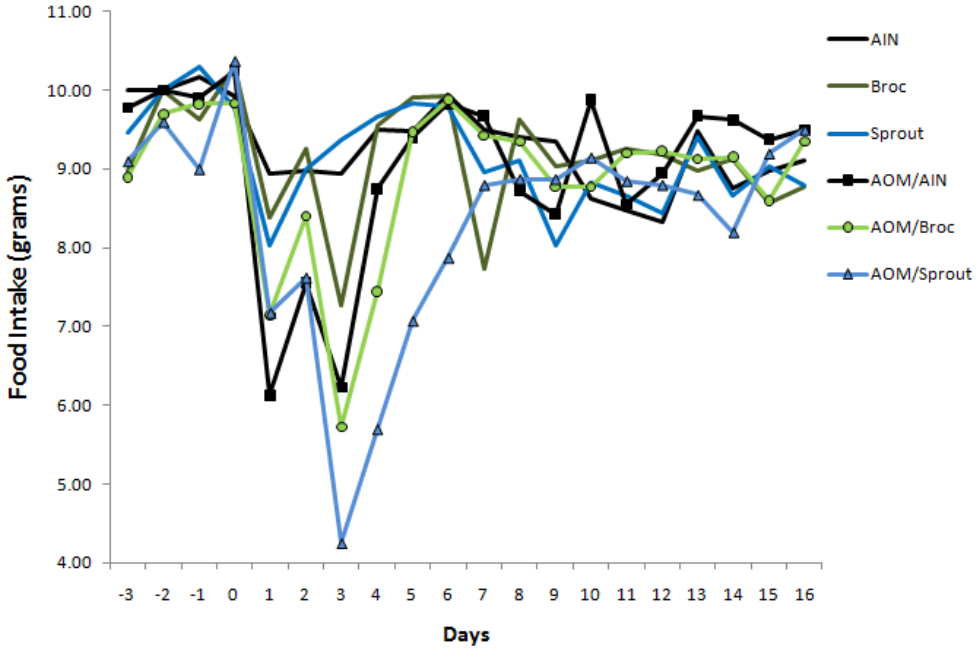
** Significantly different from AIN-93G ($p < 0.05$)

Figure 18. Food intake during AOM/DSS treatment[1]

A. Average food consumption of mice receiving control or Korean herbal diets



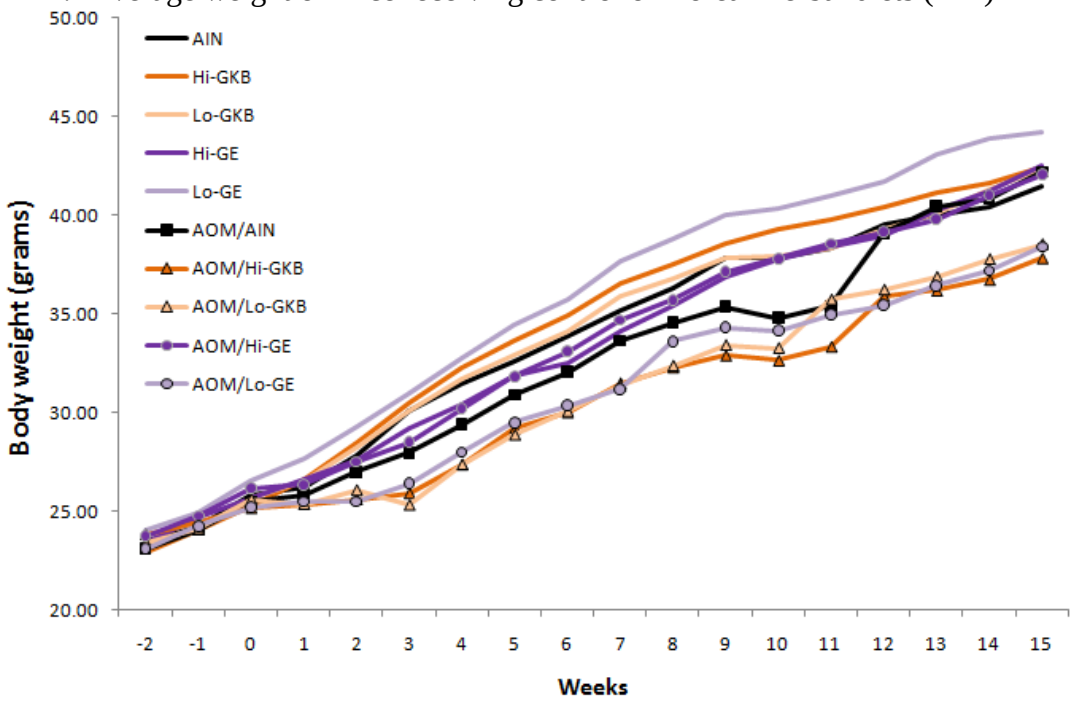
B. Average food consumption of mice receiving control or broccoli diets



[1]Food intake of mice was measured starting with the acclimation period and through AOM/DSS treatment.

Figure 19. Increase in body weights of mice, reported weekly[1]

A. Average weight of mice receiving control or Korean herbal diets (n=7)



B. Average weights of mice receiving control or broccoli diets (n=7)

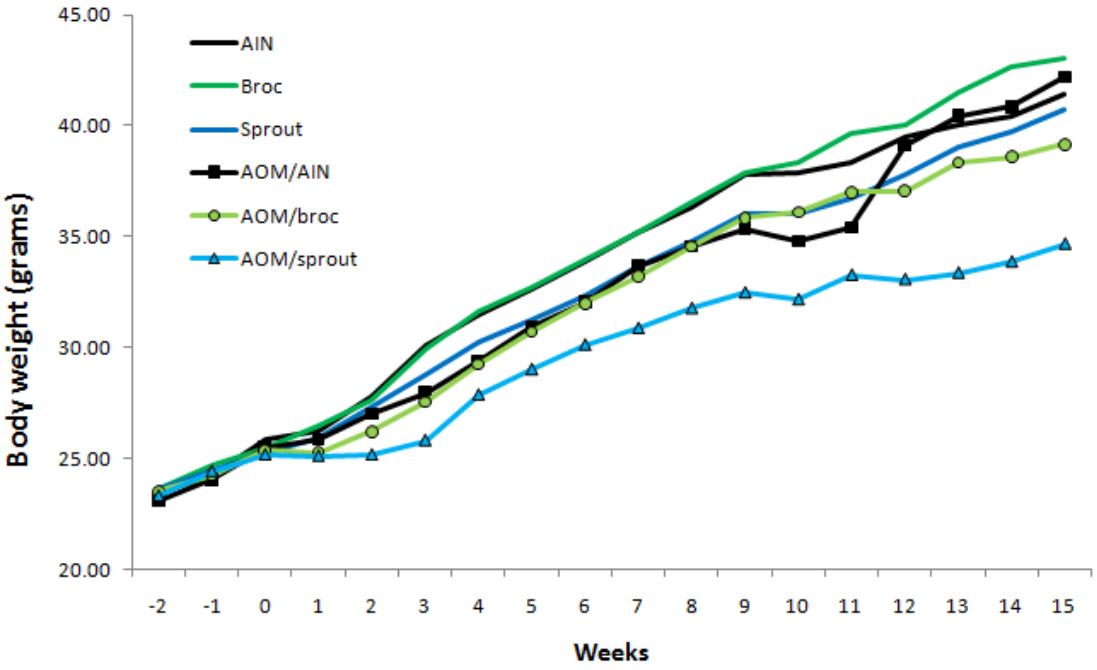
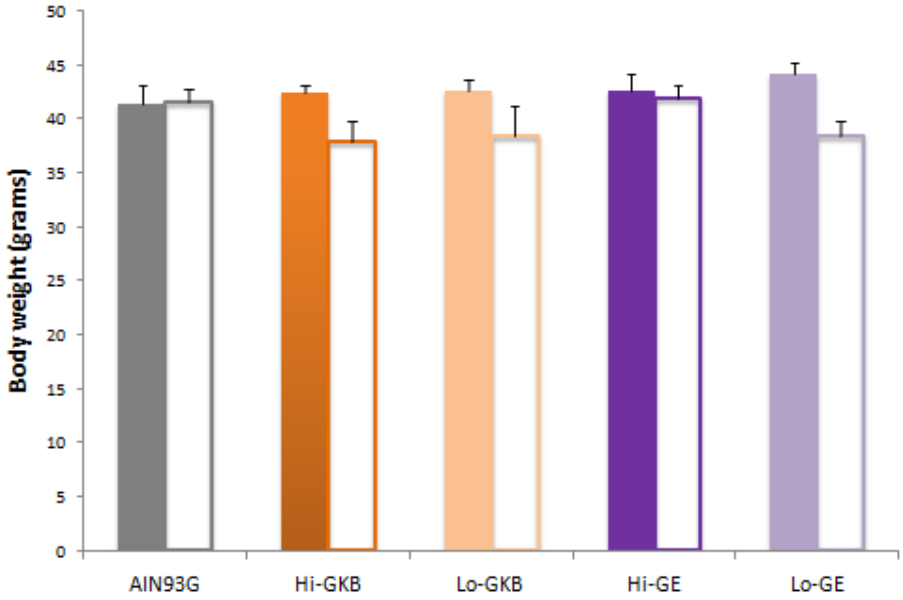
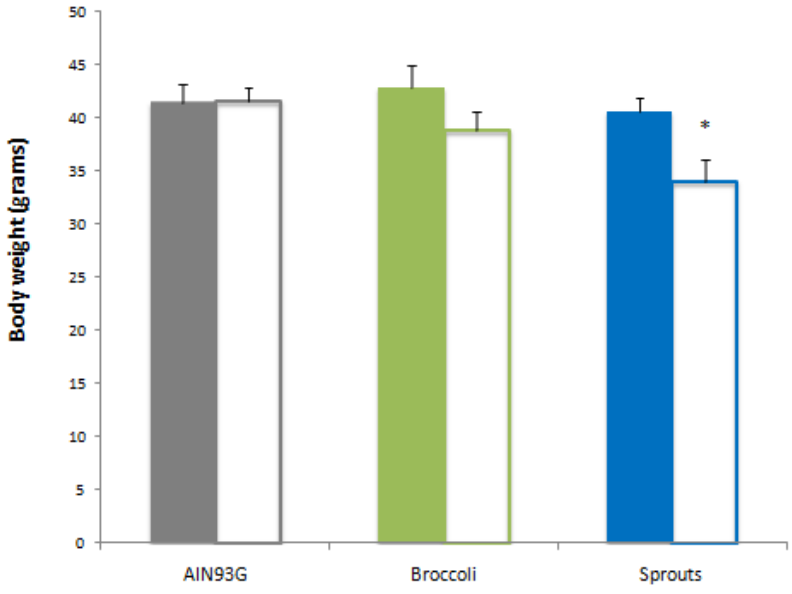


Figure 20. Final body weight measured at cull[1]

A. Final body weight of mice receiving control or Korean herbal diets (n=7)



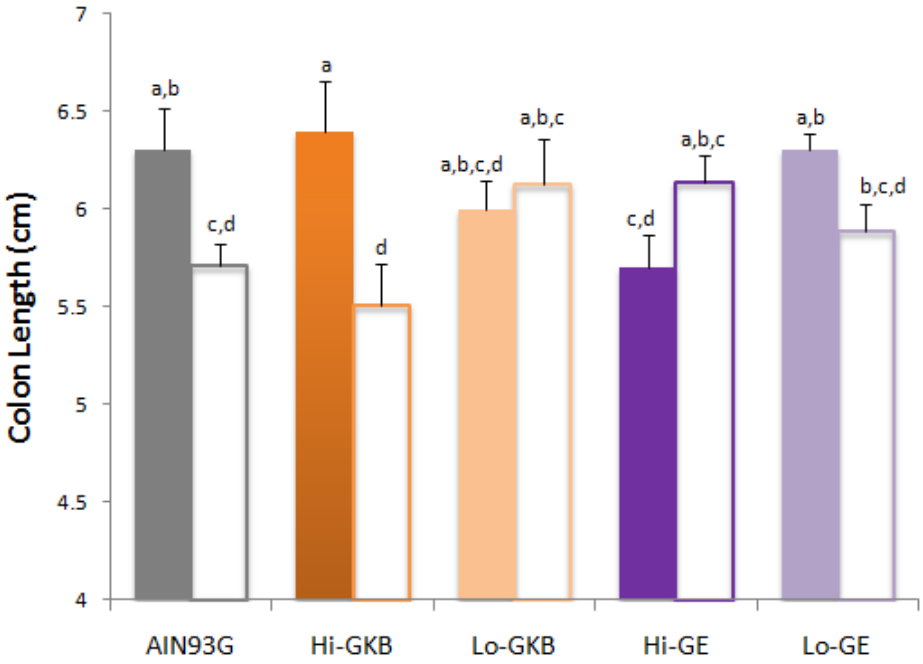
B. Final body weight of mice receiving control or broccoli diets (n=7)



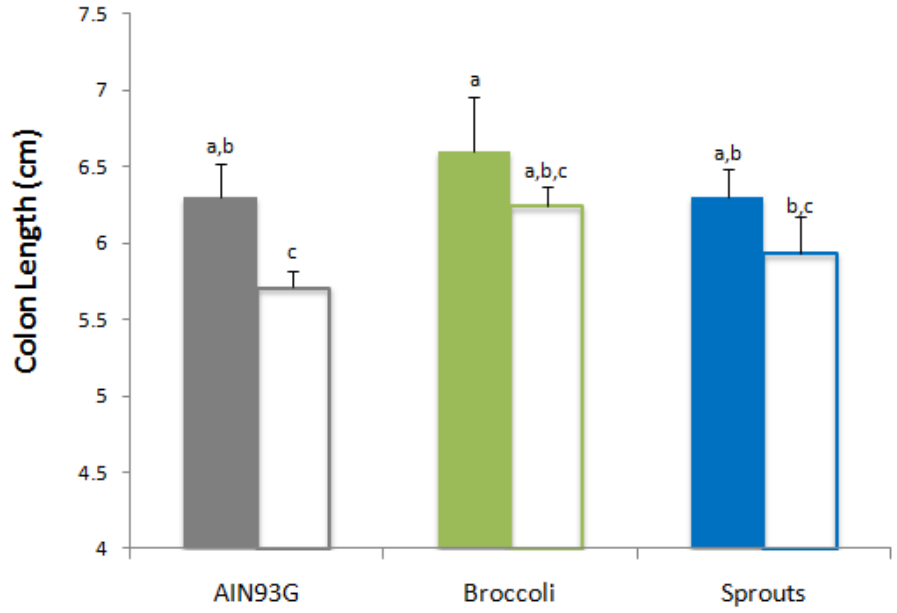
[1]Filled bars = control, open bars = AOM/DSS
Mean ± SEM, $p < 0.05$, one-way ANOVA, using Fisher's LSD
*Indicates significant difference between control and AOM treatments

Figure 21. Effect of dietary and AOM/DSS treatments on colon length[1]

A. Colon length of mice receiving control or Korean herbal diets



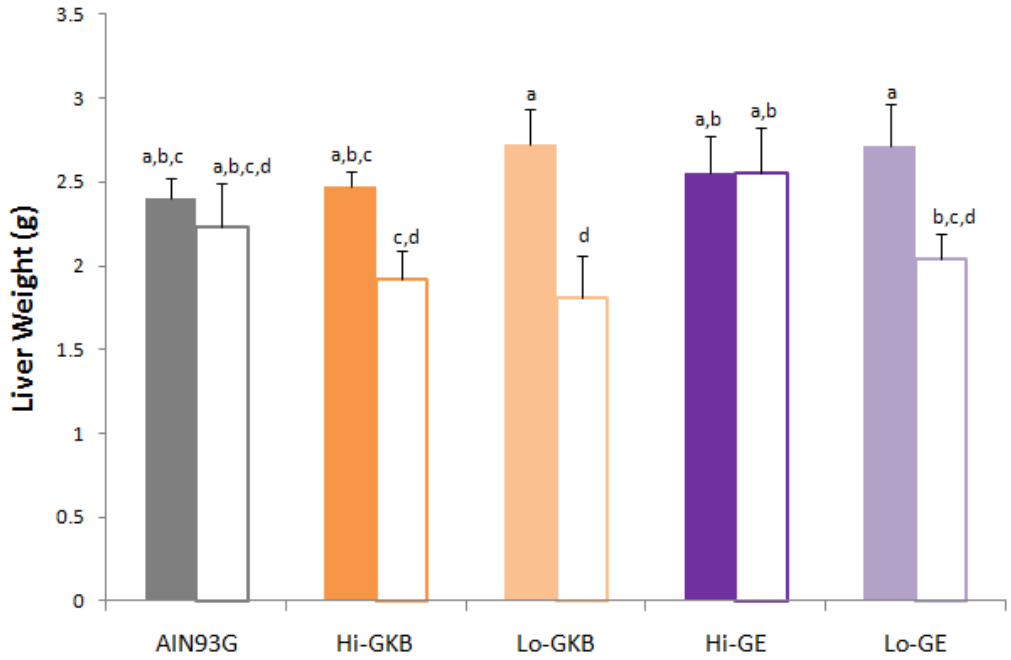
B. Colon length of mice receiving control or broccoli diets



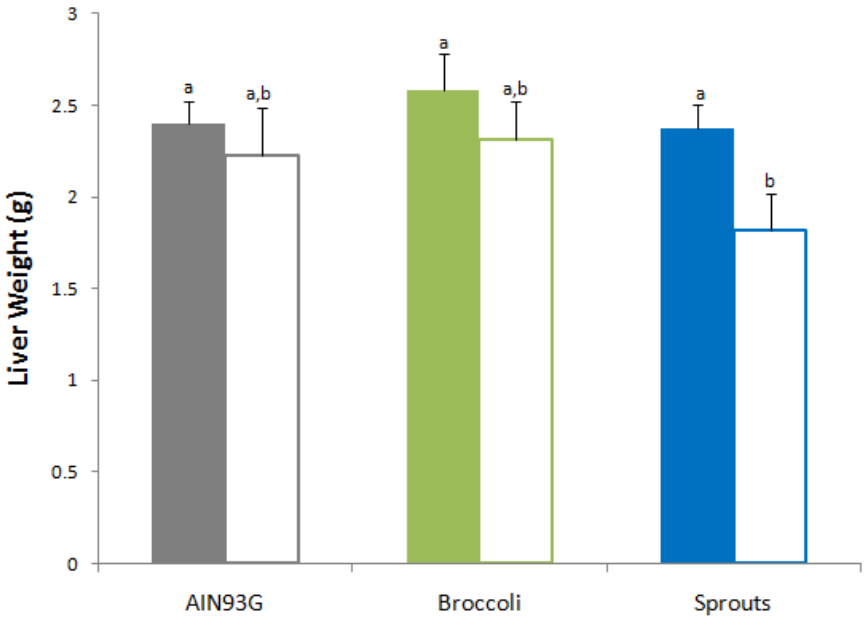
[1]Colons were removed distal from the cecum to distal from the rectum, flushed with 1.15% KCl and measured. Filled bars = control, open bars = AOM/DSS. Different letters indicate significant differences. Mean ± SEM, $p < 0.05$, one-way ANOVA, using Fisher's LSD

Figure 22. Effect of dietary and AOM/DSS treatments on liver weight[1]

A. Liver weight of mice receiving control or Korean herbal diets



B. Liver weight of mice receiving control or broccoli diets



[1]Livers were perfused with ice-cold 1.15% KCl, removed and patted dry, then weighed. Filled bars = control, open bars = AOM/DSS. Mean ± SEM, $p < 0.05$, one-way ANOVA, using Fisher's LSD. Different letters indicate significant differences

Table 11. Incidence & multiplicity of tumors in the colon of mice treated with AOM[1]

A. Effect of Korean herbal diets on tumor number

	Multiplicity (no. of tumors/mouse)			
Treatment	**Total**	**ADC[2]**	**AD[3]**	**Total**
AIN-93G	1.71 ± 0.65	1.43 ± 0.66	0.29 ± 0.2	5/7
Hi-GKB	1.29 ± 0.81	0.57 ± 0.32	0.71 ± 0.61	3/7
Lo-GKB	1.43 ± 0.32	0.86 ± 0.22	0.57 ± 0.22	6/7
Hi-GE	0.71 ± 0.31	0.14 ± 0.15*	0.57 ± 0.22	4/7
Lo-GE	1.57 ± 0.62	0.71 ± 0.31	0.86 ± 0.44	4/7

B. Effect of broccoli diets on tumor number

	Multiplicity (no. of tumors/mouse)			
Treatment	**Total**	**ADC[2]**	**AD[3]**	**Total**
AIN-93G	1.71 ± 0.65	1.43 ± 0.66	0.29 ± 0.2	5/7
Broccoli	0.71 ± 0.31	0.14 ± 0.15*	0.57 ± 0.22	4/7
Sprouts	1.00 ± 0.41	0.57 ± 0.32	0.43 ± 0.22	4/7

[1]Lesions on colon tissue from mice were identified microscopically, preserved in 10% formalin and determined to be either adenocarcinomas or adenomas by histopathology. Mean ± SEM, $p < 0.05$ using kruskal-wallis non-parametric test
[2]ADC: Adenocarcinoma [3]AD: Adenoma

Table 12. Proliferating Cell Nuclear Antigen immunohistochemistry of the colon in AOM treated mice[1]

A. Effect of Korean herbal diets on cell proliferation

	PCNA-labeling index (%)	
Treatment	**AOM (tumor fields)**	**AOM (normal fields)**
AIN-93G	82.3 ±0.03$^{a, 1}$	57.0 ±0.04^{a,2}
Hi-GKB	69.2±0.05$^{b, 1}$	54.4±0.05$^{a, 1}$
Hi-GE	70.2±0.02$^{b, 1}$	55.3±0.03$^{a, 2}$

B. Effect of broccoli diets on cell proliferation

	PCNA-labeling index (%)	
Treatment	**AOM (tumor fields)**	**AOM (normal fields)**
AIN-93G	82.3 ±0.03$^{a, 1}$	57.0 ±0.04^{a,2}
Broccoli	70.2 ±0.03^{b,1}	55.8 ±0.03^{a,2}
Sprouts	73.8 ±0.03^{ab,1}	55.1 ±0.04$^{a, 2}$

[1]Colon sections stained for Proliferating Cell Nuclear Antigen (PCNA) were used to detect % positively-stained nuclei. Tumor fields include hyperplasia within tissue, adenomas and adenocarcinomas. Normal fields include both distal and proximal sections within carcinogen treated mice. Mean ± SEM, one-way ANOVA, $p < 0.05$ using Fisher's LSD. Different letters indicate significant differences within the same column. Different numbers indicate significant differences within the same row

Table 13. Cyclooxygenase-2 immunohistochemistry of the colon in AOM treated mice[1]

A. Effect of Korean herbal diets on COX-2 induction

	COX-2 labeling index (%)	
Treatment	AOM – Tumor Fields	AOM – Normal Fields
AIN93G	$27.30 \pm 2.42^{a,1}$	$21.63 \pm 1.92^{a,1}$
Hi-GKB	$20.48 \pm 2.31^{b,1}$	$24.46 \pm 2.61^{a,1}$
Hi-GE	$15.68 \pm 1.45^{b,1}$	$23.05 \pm 1.85^{a,2}$

B. Effect of broccoli diets on COX-2 induction

	COX-2 labeling index (%)	
Treatment	AOM – Tumor Fields	AOM – Normal Fields
AIN93G	$27.30 \pm 2.42^{a,1}$	$21.64 \pm 1.92^{a,1}$
Broccoli	$17.24 \pm 3.20^{b,1}$	$20.47 \pm 1.28^{a,1}$
Sprouts	$15.32 \pm 1.28^{b,1}$	$18.78 \pm 2.12^{a,1}$

[1]Colon sections stained for COX-2 were used to detect % positively-stained nuclei. Tumor fields include hyperplasia within tissue, adenomas and adenocarcinomas. Normal fields include both distal and proximal sections within carcinogen treated mice. Mean ± SEM, one-way ANOVA, n=7, $p < 0.05$ using Fisher's LSD and Student's T-test between normal and tumor fields. Different letters indicate significant differences **within the same column.** Different numbers indicate significant differences **within the same row.**

Table 14. Measurement of serum IL-6 by ELISA[1]

A. Effect of Korean herbal diets on induction of interleukin-6 in the serum

	Serum IL-6 concentration (pg/mL)	
Treatment	**Control**	**AOM/DSS**
AIN-93G	$3.24 \pm 0.99^{a,1}$	$33.85 \pm 5.00^{a,2}$
Hi-GKB	$5.83 \pm 0.62^{a,1}$	$24.59 \pm 8.17^{ab,1}$
Lo-GKB	$4.48 \pm 0.83^{a,1}$	$25.47 \pm 7.51^{ab,1}$
Hi-GE	$5.88 \pm 0.90^{a,1}$	$19.40 \pm 3.21^{b,2}$
Lo-GE	$4.94 \pm 0.27^{a,1}$	$20.12 \pm 2.94^{b,2}$

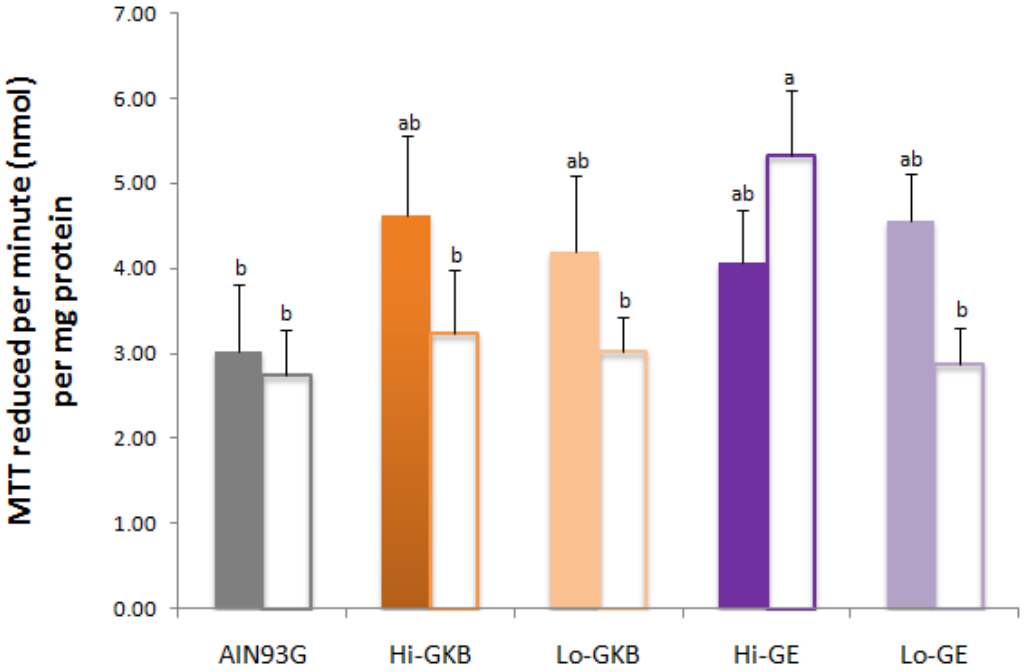
B. Effect of broccoli diets on induction of interleukin-6 in the serum

	Serum IL-6 concentration (pg/mL)	
Treatment	**Control**	**AOM/DSS**
AIN-93G	$3.24 \pm 0.99^{b,1}$	$33.85 \pm 5.00^{a,2}$
10% Broccoli	$7.56 \pm 0.78^{a,1}$	$15.91 \pm 4.20^{b,1}$
5% Sprouts	$7.82 \pm 0.52^{a,1}$	$16.65 \pm 6.29^{b,1}$

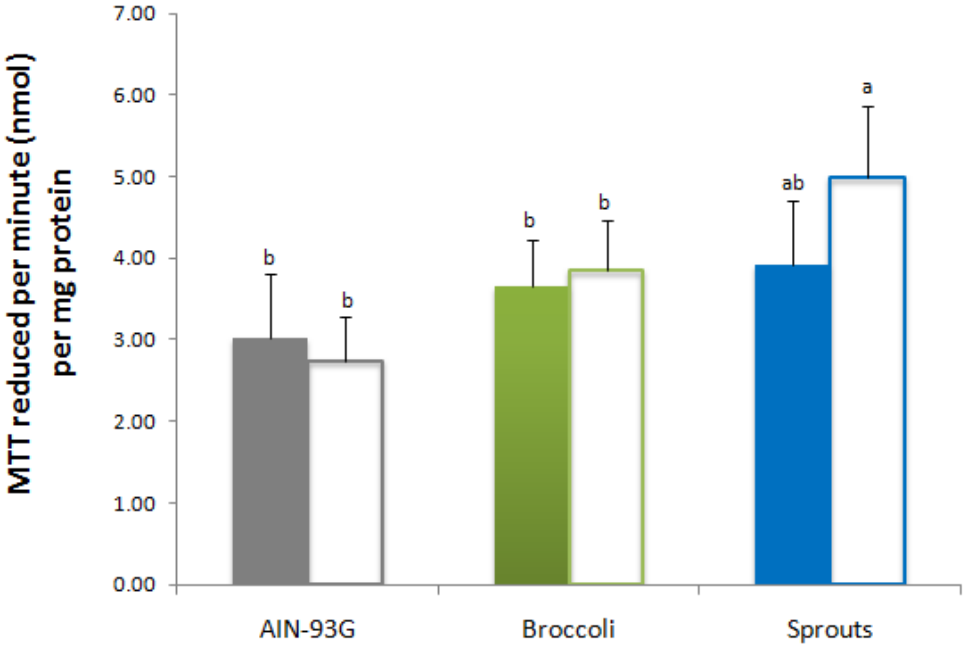
[1]Serum collected at the end of the study was assayed for IL6 by enzyme-linked immunosorbentassay (ELISA). Mean $\pm$ SEM, n=7, one-way ANOVA, $p < 0.05$ using Fisher's LSD and Student's t-test between control and AOM/DSS. Different letters indicate significant differences **within the same column.** Different numbers indicate significant differences **within the same row.**

Figure 23. Induction of NAD(P)H-quinone oxidoreductase-1 (NQO1) activity[1]

A. Effect of Korean herbal diets on QR enzyme induction in liver



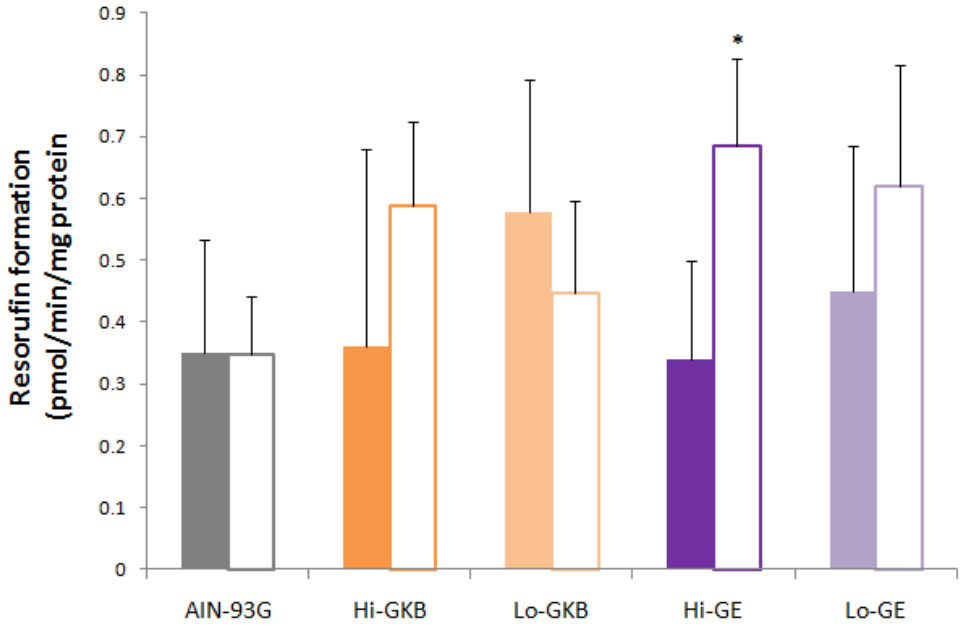
B. Effect of broccoli diets on QR enzyme induction in liver



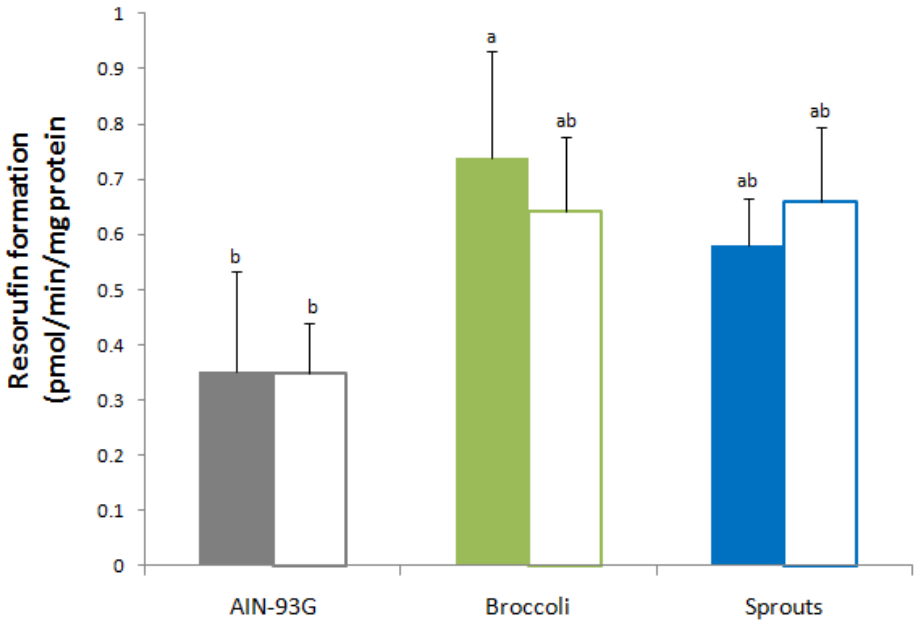
[1]Activity of cytosolic NQO1 activity was measured in liver. Filled bars = control, open bars = AOM/DSS. Mean ± SEM, one-way ANOVA, n=7, $p < 0.05$ using Fisher's LSD and Student's T-test. Different letters indicate significant differences between all bars.

Figure 24. Induction of ethoxyresorufin-O-deethylase (EROD) activity[1]

A. Effect of Korean herbal diets on EROD enzyme induction in liver microsomes



B. Effect of broccoli diets on EROD enzyme induction in liver microsomes



[1]Activity of CYP1A by microsomal EROD enzyme induction was measured in the liver. Filled bars = control; open bars = AOM/DSS. Mean ± SEM, one-way ANOVA, n=7, $p < 0.05$ using Fisher's LSD and Student's T-test. Different letters indicate significant differences between all bars.
*Indicates significant difference between filled and unfilled bars within the same treatment group. Data were normalized using log transformation.

Table 15. Standard AIN-93G diet composition
Gymnaster Extract and Gymnasterkoreayne B were added to the soybean oil before mixing

Formula (1 Kg diet)	AIN-93G (g/Kg)
Casein	200.0
Celluose	50.0
Corn Starch	397.486
Maltodextrin	132.0
Sucrose	100.0
L-Cystine	3.0
Mineral Mix	35.0
Vitamin Mix	10.0
Choline Bitartrate	2.5
Soybean Oil	70

Table 16. Broccoli sprouts diet calculation

Formula (1 Kg diet)	AIN-93G (g/Kg)	5% Broccoli sprouts
Sprouts	--	50.0
Casein	200.0	176.64
Celluose	50.0	31.64
Corn Starch	397.486	397.5
Maltodextrin	132.0	129.15
Sucrose	100.0	97.84
L-Cystine	3.0	3.0
Mineral Mix	35.0	31.66
Vitamin Mix	10.0	10.0
Choline Bitartrate	2.5	2.5
Soybean Oil	70	70

Broccoli sprouts, raw
(source: http://www.broccosprouts.com/health/nutricontent.htm)

Component	Value/100g fresh weight (DW=10.7g)	50g dry weight *
Water	89.29	
Energy	57.14 kcal	
Protein	5.0	23.63 (example: 5.0*50/10.7)
Total fat	0	0
Ash (total mineral)	.71	3.33
Carbohydrate (+fiber)	6.79	31.71
Fiber (total dietary)	3.93	18.34
Sugars (total)	1.07	5.0

*These values were used to calculate 5% dry weight broccoli sprouts in the diet.

Adjusted AIN-93G diet as follows:
Subtract protein from casein
Subtract total lipid from soybean oil
Subtract ash from mineral mix
Subtract starch portion of carbohydrate from corn starch
Subtract dietary fiber from cellulose
Subtract ratio of sugar from maltodextrin and sucrose (calculated below)

Maltodextrin	**132**	**2.85 (=5.00*132/232)**
Sucrose		**100**	**2.16 (=5.00*100/232)**
Total sugar (sprouts)		**232**	**5.00**

CHAPTER 6
CONCLUSION

Initial studies (chapters 2 and 3) showed that DSS-induced inflammation in mice is a good model to test potential plant bioactive compounds as anti-inflammatory agents. We also observed that rappini and broccoli can protect against DSS (1%) induced shortening of the colon in mice. Our question then became whether or not broccoli, or other plant bioactive compounds, could protect against inflammation-enhanced colon cancer in mice.

The most important findings from this research are that broccoli and the GKB-rich extract from *Gymnaster koraiensis* (GE) are able to protect both against some aspects of inflammation and slow the progression of colitis associated colon cancer, and against chemical-induced carcinogenesis of the colon. Further work is needed to determine if the reason for greater efficacy of the GKB-rich extract compared to purified GKB is due to an additional active component(s) or to improved bioavailability of GKB. Possibly the substantial positive effect of the high dose of the extract in slowing the progression of benign adenomas to malignant adenocarcinomas may be due in part to its ability to decrease inflammation.

Broccoli sprouts are higher in sulforaphane (7.66μmol/g DW) than broccoli (0.72μmol/g DW); therefore we expected that broccoli sprouts would be the positive control to study the effect of sulforaphane from whole broccoli. Sulforaphane is a known inducer of apoptosis in human colon cancer cells (137). A recent study showed that sulforaphane induces autophagy in colon cancer cells and that by inhibition of autophagy, that the pro-apoptotic effect of sulforaphane is heightened (138). The clinical significance of this study is that specific cellular mechanisms can be targeted during cancer treatment. Specifically, autophagy inhibitors may be useful by increasing the effectiveness of chemopreventive treatments.

However, in this study, broccoli was significantly more effective than broccoli sprouts in preventing the development of colon tumors from benign to malignant. This suggests that broccoli may contain a more desirable profile of bioactive components than broccoli sprouts and that sulforaphane may not be the most important bioactive to protect against tumor formation in the colon. Further investigation is needed into the mechanisms by which broccoli and its bioactive components can protect against inflammation and colon cancer.

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